



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C07K 14/00</b>		<b>A2</b>	(11) International Publication Number: <b>WO 99/03884</b>
			(43) International Publication Date: 28 January 1999 (28.01.99)
(21) International Application Number: PCT/US98/14716 (22) International Filing Date: 21 July 1998 (21.07.98) (30) Priority Data: 60/053,306                      21 July 1997 (21.07.97)                      US 60/073,456                      2 February 1998 (02.02.98)                      US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US                                      60/053,306 (CIP) Filed on                                      21 July 1997 (21.07.97) US                                      60/073,456 (CIP) Filed on                                      2 February 1998 (02.02.98) (71) Applicant (for all designated States except US): NORTH AMERICAN VACCINE, INC. [CA/US]; 12103 Indian Creek Court, Beltsville, MD 20705 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MINETTI, Conceicao [BR/US]; 3904 Isbell Street, Silver Spring, MD 20306 (US). MICHON, Francis [CA/US]; 4401 Rosedale Avenue, Bethesda, MD 20814 (US). PULLEN, Jeffrey, K. [US/US]; 6928 Garland Lane, Columbia, MD 21045 (US).		POLVINO-BODNAR, Maryellen [US/US]; 621 Rolling Dale Road, Annapolis, MD 21401 (US). LIANG, Shu-Mei [US/US]; 6627 River Road, Bethesda, MD 20817 (US). TAI, Joseph, Y. [US/US]; 1370 Cinnamon Drive, Port Washington, PA 19034 (US). (74) Agents: RADY, Arnold, I. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published Without international search report and to be republished upon receipt of that report.	

## (54) Title: MODIFIED IMMUNOGENIC PNEUMOLYSIN COMPOSITIONS AS VACCINES

ATGCGAATA AAGCAGTAA TGACTTTATA CTAGCTATGA	40	ACACGAGATC ATCCAGGCTT GCGATTTTCC TATACGACTT	1000
ATTACGATAA AAGAAACTC TTGACCCATC AGGAGAAAG	80	CTTTTITAGS TGCAATGTA GTTTCGACCT TTCAAAATAG	1040
TATTAATAAT GTTTTCATCA AAGAGGCTAA TCACTACCC	120	TACGACTAT GTTGAGACTA AGGTTACAGC TTACAGAAAC	1080
GATGATTTTG TTGTTATGCA AAGAAAGAG CGAGCTTTGT	160	GGAGATTAC TGTGATCA TAGTGGTGGC TATGTTGGCC	1120
CGAAGATAC AAGTGAATAT TCTGTAAAGG CTACCAACA	200	AATATTATAT TACTTGAAT GAATTATCCT ATGATCATCA	1160
CAGTGGCTTC TATCTGGAG CACTTCTGCT AGTGGATGAG	240	AGGTAAAGAA GTCTTGACTC CTAAAGCTTG GGACAGAAAT	1200
ACCTTTTATG AAGATTAATC CACTTCTCTT GCGGTGATC	280	GGCAGAGATT TAACGGCTCA CTTTACCACT AGTATCTCTT	1240
GTGCTGGAT GACTTATAGT ATTGATTTTC GTGGTTGGC	320	TAAAGGGAAA TGTGTAAT CTCTCTGTCA AATATGAGA	1280
AAGTAGCAT AGCTTCTTC AAGTGAAGA TCCAGCAAT	360	GTGTACCGGG CTGCTGGG AATGGTGGG TACGGTTAT	1320
TCAAGTCTTC GCGAGCGGT AAACGATTTG TTGCTAAGT	400	GAAGAAACCG ATTTCGACT AGTGGTAGG CGAGGATTT	1360
GGATCAAGA TTATGGTCAG GTCAATATG TCCAGCTAG	440	CTATTGGGG ACAACTCTC TATCCGAGG TAGAGATAA	1400
AATCGCATAT GAAGAAATCA CGGCTCAGG CATGGAACA	480	GGTAGAAAT GAC	1413
CTCAAGTCA AGTTTGGTTC TGACTTTGAA AAGCAGGGA	520		
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ACAGTCAGG TAGAGCGTGT TAAAAATCCA GGAGATGTGT	640		
TTCAAGATAC TGTAAAGGTA GAGGATTTAA AACGAGAGG	680		
AATTCTGCA GAGCTCTCTT TGGTATATAT TTGAGTGT	720		
GCTTATGGC GCGAGTCTA TCTCAAGTTG GAAGCAAGA	760		
GTAAAGTGA TGAATGAGG GCTGCTTTTG AAGCTTTGAT	800		
AAGAGAGTC AAGGTAGCTC CTCAGACAGA GTGAGACAG	840		
ATTTTGAACA ATACAGAGAT GAAGCGGCTT ATTTTGAAGG	880		
GGACCCAGG TTGCGGTGCC CGAGTTGTAA CAGCAAGGT	920		
GGATATGTA GAGGACTTGA TCAAGAGG CAGTCTCTT	960		

A

B

## (57) Abstract

This invention relates to modified pneumolysin polypeptides that retain the immunogenic nature of pneumolysin but have reduced or undetectable hemolytic activity compared to native pneumolysin. The invention also provides a method for generating novel pneumolysin variants with these desired characteristic properties. The invention also provides immunogenic compositions useful as pharmaceutical compositions including vaccines in which non-toxic, modified pneumolysin is used to stimulate protective immunity against *Streptococcus pneumoniae*. The vaccines may be compositions in which the modified pneumolysin is conjugated to bacterial polysaccharides or may be carried on an attenuated viral vector. In addition, the invention also provides a method of using the non-toxic, modified pneumolysin toxoid in order to stimulate antibodies against *Streptococcus pneumoniae* in a treated individual which are then isolated and transferred to a second individual, thereby conferring protection against *Streptococcus pneumoniae* in thesecond individual.

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**MODIFIED IMMUNOGENIC PNEUMOLYSIN COMPOSITIONS AS VACCINES****FIELD OF THE INVENTION:**

This invention relates to the field of vaccines, and in particular, methods for the production of modified forms of pneumolysin and their use in producing compositions for the immunization of mammals against infections caused by bacteria including *Streptococcus pneumoniae*.

**BACKGROUND OF THE INVENTION:**

*Streptococcus pneumoniae* is the major cause of bacterial pneumonia, bacteremia, meningitis, and otitis media (Baltimore et al. in *Bacterial infections of humans: Epidemiology and control* Evans and Brachman eds, Plenum Press, New York, 1989 pp.525-546; Schuchat et al. *N. Engl. J. Med.* 1997, 337, 970-976). Even with appropriate antibiotic therapy, pneumococcal infections have been estimated to result in as many as 40,000 deaths a year in the United States (Fedson et al. *Archives of Internal Medicine* 1994, **154**, 2531-2535; Fiebach et al. *Archives of Internal Medicine* 1994, **154**, 2545-2557). In addition, pneumococci have gained increased resistance to penicillin and other antibiotics making the development of an effective vaccine to prevent pneumococcal infections a public health priority (Farr et al. *Archives of Internal Medicine* 1995, **155**, 2336-2340). Since the current 23-valent pneumococcal capsular polysaccharide vaccine is ineffective in children less than two years old (Douglas et al. *J Infect Dis* 1983, **148**, 131-137; Leinonen et al.

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*Pediatric Infectious Disease Journal* 1986, **5**, 39-44), numerous groups are developing multivalent conjugate vaccines to prevent otitis media, the major indication in this age group.

5                   Pneumolysin (PLY), a sulfhydryl-activated cytolytic toxin, is produced by all types of *Streptococcus pneumoniae* (Kancierski et al. *J Clin Microbiol* 1987, **25**, 222-225) and is considered a major virulence factor in pneumococcal infection (Boulnois *Journal of General*  
10 *Microbiology* 1992, **138**, 249-259). Genetically engineered PLY-negative mutant strains of *S. pneumoniae* have been shown to be significantly less virulent in mice (Berry et al. *Microb Pathog* 1992, **12**, 87-93; Berry et al. *Infection and Immunity* 1989, **57**, 2037-2042). Cytotoxicity of PLY to  
15 pulmonary endothelial and epithelial cells is well demonstrated in vitro (Rubins et al. *Infection and Immunity* 1992, **60**, 1740-1746). In addition, PLY may be the principal cause of hearing loss and cochlear damage in a guinea pig model of pneumococcal meningitis (Winter et  
20 al. *Infection and Immunity* 1997, **65**, 4411-4418).

                  As of 1985, an estimated five million children under the age of 5 died from pneumonia caused by *S. pneumoniae* in developing countries each year. *Lancet* (1985) Sep 28 **2(8457)**:699-701. *S. pneumoniae* employs a  
25 number of virulence factors to establish an initial infection and then produce invasive disease(s). To prevent systemic infections caused by the various serotypes of *S. pneumoniae*, immunization of infants and adults with suitable, cross-reactive vaccines, capable of  
30 eliciting safe, effective, and long-lasting immunity, is

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needed.

In a prospective study of pneumococcal colonization and infection in children, it was reported that pneumococcal serotypes 6, 14, 19, and 23 are the most commonly carried as well as the most frequent cause of infection in infants, mainly otitis media (Gray et al. *J. Infect. Dis.*, 1988, 158, 948-955). In addition, it was recently found that these same strains are more frequent among the penicillin resistant clinical isolates (Nesin et al. *J. Infect. Dis.*, 1998, 177, 707-713). Clinical studies carried out in young infants with a tetravalent pneumococcal conjugate vaccine including the above types, report a reduction in the carriage of vaccine-related strains (Dagan et al. *Infect. Dis. J.*, 1997, 16, 1060-1064).

Almost all isolates of *S. pneumoniae* exhibit an external capsule made up of repeating oligosaccharides. Antigenic differences in the capsular polysaccharides due to different saccharide sequences are the hallmark of the different *S. pneumoniae* serotypes. Serotype-specific capsular polysaccharides are the major contributors to the virulence of the pneumococcus. Existing anti-pneumococcal vaccines are formulated from 23 capsular polysaccharides selected from the 84 serologically distinct types currently recognized. Unfortunately, these vaccines are not effective in all populations, especially those of Asia. A second shortcoming of the current vaccines is that polysaccharides by themselves are poor immunogens, especially for infants and the elderly.

Polypeptides expressed by *S. pneumoniae* also

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play an important pathogenic role. Some of the defined polypeptides that appear to contribute to the virulence of this organism include pneumolysin, autolysin, neuraminidase, pneumococcal surface polypeptide A (PspA), the 37 kDa polypeptide, adhesion molecules, hyaluronidase, and an IgA1 protease.

Virtually all serotypes of *S. pneumoniae* produce pneumolysin, one of the major virulence factors. This expression by the various *S. pneumoniae* serotypes makes pneumolysin a prime candidate for use in a protective vaccine against pneumococcal infections provided its toxicity can be altered.

Pneumolysin is an intracellular bacterial polypeptide with a molecular weight of approximately 53-kD. (KancIerski et al. (1987) *J. Clin. Microbiol.* **25**:222-225.) It is a member of a family of thiol-activated hemolysins and has various effects on eukaryotic cells. Pneumolysin is known to bind to cholesterol molecules in the eukaryotic membrane, form oligomers, and generate transmembrane pores. It has also been demonstrated that the respiratory burst, chemotactic, and phagocytic functions of polymorphonuclear leukocytes, all of which are critically important for removing invading pneumococci, are severely compromised in the presence of pneumolysin.

Pneumolysin causes both cytolytic and cytotoxic effects, and can stimulate an inflammatory response by the complement activation pathway. Nonspecific activation of complement causes depletion of complement polypeptides and generates nonspecific inflammation. Inoculation of

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pneumolysin into lungs of experimental animals causes pneumoniae-like symptoms. However, pre-immunization with pneumolysin is protective for experimental animals upon challenge with pneumococci. Paton *et al.* (1983) *Infect. Immun.* **40**:548-552.

Because of pneumolysin's immunogenic activity and capacity to elicit a protective response in individuals immunized with it, it has been suggested to use pneumolysin as a component of a vaccine. See PCT/AU89/00539. However, before pneumolysin can be included in vaccines for human use, this toxin must be modified so as to be substantially non-toxic while retaining the capacity to elicit protective antibodies.

Modified pneumolysins devoid of toxic activities are reported to have been generated based on the identification of amino acid regions of pneumolysin thought to have similar functions to related thiol-containing polypeptides. (WO 90/06951). The reported mutations are exclusively in the C-terminal portion of the polypeptide and were generated using targeted mutagenesis techniques. Other mutations, including certain specific amino acids in the N-terminal region have been reported to reduce hemolytic activity. The most significant reduction in hemolytic activity is reported as possibly being a result of histidine modification at position 156. Hill *et al.* (1994) *Infection and Immunity*, 62, 757-758. No data is provided concerning whether any of these substituted pneumolysins were properly refolded. A single mutation, Thr-172→Ile was reported to be responsible for a pneumolysin with reduced hemolytic activity. However,

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anomalous electrophoretic mobility indicates that the protein is incorrectly folded. Lock et al. *Microb. Pathog.* (1996) 21, 71-83.

#### SUMMARY OF THE INVENTION:

5           This invention provides a novel method for generating and identifying stable, genetically modified, substantially non-toxic, immunogenic pneumolysin polypeptides using random PCR mutagenesis. Modified pneumolysin (pneumolysoid) which can be used as immunogens  
10   in a vaccine or can be used as an immunogenic carrier polypeptide for polysaccharide conjugate vaccines against *S. pneumoniae* or other bacterial infections are also provided. The modified pneumolysin polypeptides of this  
15   invention, while exhibiting substantially reduced or none of the toxin's toxic activity, elicit antibodies which are cross-reactive with those elicited by the native toxin.

          This invention also relates to nucleic acid sequences encoding the modified pneumolysins, vectors containing them as well as transformed host cells capable  
20   of expressing the nucleic acid molecules of this invention.

          Another embodiment of this invention is polysaccharide-polypeptide conjugate molecules in which the modified pneumolysin of this invention is covalently  
25   coupled to bacterial polysaccharide to form the conjugate. Such conjugate molecules are useful as immunogens for eliciting a T cell dependent immunogenic response directed against the bacterial polysaccharide conjugated to the modified pneumolysin.



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The invention is further directed to pharmaceutical compositions containing the modified pneumolysin polypeptides of the invention which elicit an immune response.

5           This invention further relates to a method of eliciting the production of antibodies reactive to the modified pneumolysin polypeptides. Such antibodies may be used to elicit both active and passive immunity. The modified pneumolysins of this invention may also be used  
10       to identify and isolate reactive antibodies.

          It is therefore an object of this invention to provide genetically stable, modified *S. pneumoniae* pneumolysin polypeptides which have substantially attenuated or absent toxicity while retaining epitopes  
15       which cause production of antibodies which also bind the native toxin molecule.

          It is a further object of this invention to provide a method for generating genetically modified pneumolysins(pneumolysoids).

20           It is another object of this invention to provide vaccine preparations comprising a modified pneumolysin polypeptide that can elicit antibodies and induce protective immunity against *Streptococcus pneumoniae* when delivered to a susceptible mammal. Such  
25       vaccines may be based on the pneumolysoid itself, or conjugates that comprise one or more bacterial polysaccharides covalently bound to a modified pneumolysin polypeptide of this invention.

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**BRIEF DESCRIPTION OF THE DRAWINGS:**

FIG. 1: Wild-type nucleic acid sequence of type 14 pneumolysin.

FIG. 2: Non-limiting nucleic acid variations of type 14 pneumolysin. The residue position followed by examples of nucleic acid substitutions that attenuate hemolytic activity are: 181, C; 443, A; 583, A or G. The residue position followed by examples of nucleic acid substitutions not observed to attenuate hemolytic activity are: 50, G; 54, T; 98, C; 122, G; 134, C; 137, C; 187, T; 196, T; 248, C; 276, C; 302, C; 305, G; 351, T; 380, A; 382, C; 459, C; 514, G; 558, C; 566, G; 717, A; 764, G; 770, G; 1038, T; 1138, A; 1212, A; 1296, T; 1386, G; 1395, A.

FIG. 3: Amino acid sequence of type 14 pneumolysin.

FIG. 4: Non-limiting amino acid variations of type 14 pneumolysin. The residue position followed by examples of amino acid substitutions that attenuate hemolytic activity are: 61, Pro; 148, Lys; 195, Ile or Val; 243, Arg, Val, Glu, or Ser; 286, Asp; 446, Ser. The residue position followed by examples of amino acid substitutions not observed to attenuate hemolytic activity are: 17, Arg; 18, Asn; 33, Thr; 41, Gly; 45, Ala; 46, Thr; 63, Ser; 66, Tyr; 83, Ser; 101, Thr; 102, Gly; 127, Glu; 128, His; 153, Met; 172, Ala; 189, Arg; 239, Arg; 255, Gly; 257, Gly.

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FIG. 5: Map of plasmid pNV-19 containing wild-type pneumolysin nucleic acid sequence. The pNV series of plasmids were derived from pET-24a by cloning in modified pneumolysin nucleic acid sequences.

5                   FIG. 6: Diagram showing the positions of the nucleic acid and amino acid substitutions in specific modified pneumolysin polypeptides pNVJ1, pNVJ45, pNVJ20, pNVJ22, pNVJ56, pNV103, pNV207, pNV111, pNV211.

10                   FIG. 7: SDS-PAGE showing expression of recombinant pneumolysin following IPTG induction.

FIG. 8: Comparison of polysaccharide dose response of polysaccharide specific IgG following two injections of monovalent or tetravalent pneumococcal pneumolysoid vaccines in mice.

15                   FIG. 9: Comparison of polysaccharide-specific IgG following two injections in mice of tetravalent pneumococcal vaccines conjugated to pneumolysoid or tetanus toxoid carriers.

20                   FIG. 10: Pneumolysoid-specific IgG elicited by monovalent and tetravalent pneumococcal polysaccharide-pneumolysin vaccines in mice after two injections.

25                   FIG. 11: Polysaccharide-specific opsonophagocytic activity elicited by tetravalent pneumococcal PS-pneumolysoid and PS-tetanus toxoid conjugate vaccines in mice after two injections.

FIG. 12: Anti-hemolytic pneumolysoid-specific

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activity elicited by monovalent and tetravalent pneumococcal conjugates in mice after three injections.

FIG. 13: Hemolysis Inhibition Assay. Hemolysis titer of wild type pneumolysin upon pre-incubation with the indicated mutants. The bars represent the final hemolytic titer of the wild type tested against erythrocytes pre-treated with the indicated mutants.

FIG. 14: Competitive inhibition ELISA studies between a rabbit polyclonal antibody to wild type PLY and wild type PLY protein using soluble wild type PLY, PLYD mutant pNV207 (A) and PLYD mutant pNV103 (B).

FIG. 15: Fluorescence Spectra of Wild Type Pneumolysin and Mutants. Fluorescence emission spectra of wild type pneumolysin and selected mutants recorded in 10 mM sodium phosphate (pH 7.5) employing an excitation wavelength of 290 nm and monochromator slits of 2 nm. ○ represents pNV207, ● represents pNV111, ◇ represents pNV211, + represents pNV103, and □ represents wild-type.

FIG. 16: (A) Far UV CD spectra of mutant pneumolysin pNV207 (upper chart) and type 14 CPS conjugated mutant pneumolysin pNV207 (lower chart); (B) near UV CD spectra of mutant pneumolysin pNV207 (upper chart) and type 14 CPS conjugated mutant pneumolysin pNV207 (lower chart).

FIG. 17: (A) Tetravalent pneumococcal pneumolysoid pNV207 conjugate vaccine in mice: polysaccharide-specific IgG response over time; (B) tetravalent pneumococcal TT conjugate vaccine in mice:

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polysaccharide-specific IgG response over time; (C) monovalent pneumococcal pneumolysoid pNV207 conjugate vaccines in mice: polysaccharide-specific IgG response over time.

5                   **DETAILED DESCRIPTION OF THE INVENTION:**

Pneumolysin is found in virtually all known strains of *S. pneumoniae*. Its broad distribution provides the ability to obtain substantial cross-protection among different *S. pneumoniae* serotypes. This invention  
10 provides genetically modified pneumolysin polypeptides which act as toxoids (pneumolysoids) and are therefore useful for eliciting antibodies and for use in vaccines against *S. pneumoniae*. Nucleic acid sequences encoding the modified pneumolysins, vectors and host cells  
15 transformed with vectors comprising the nucleic acids encoding the modified pneumolysins are also embodiments of this invention.

The modified pneumolysin polypeptides of this invention in which at least one amino acid is substituted,  
20 retain sufficient epitopes to be immunogenic and elicit antibodies which are cross-reactive with wild-type pneumolysin. In addition, the toxicity of such modified polypeptides is sufficiently reduced to allow for their administration to mammals without substantial risk of  
25 dangerous side effect.

In an embodiment of this invention, specific modified pneumolysin polypeptides are provided which are covalently bound to polysaccharides to produce conjugates. By conjugating the modified pneumolysin polypeptides of

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this invention to different polysaccharides, this invention provides compositions capable of eliciting antibodies to a wide range of serologically distinct pathogens. By selecting the capsular polysaccharide from specific bacteria, this invention can be used to provide immunization against meningococcus, pneumococcus, haemophilus influenzae type b and Group B streptococcus as well as other bacteria.

In another embodiment of the invention, genetic modifications in the pneumolysin genome are generated using random mutagenesis techniques.

**A. Method For Producing and Identifying Modified Pneumolysin**

Genetically modified pneumolysin polypeptides of this invention are produced using conventional recombinant methodology (Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* 2nd ed., Cold Spring Harbor Laboratory Press and Ausubel et al. Eds. (1997) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). Minor variant forms of pneumolysin polypeptides have been reported which show high degrees of conservation of amino acid and nucleic acid sequences. See, for example, Mitchell et al. (1990) *Nucleic Acid Res.* **18**:4010 which is incorporated herein by reference and which reports that isoleucine at position 153 of pneumolysin of type 1 *S. pneumoniae* is substituted with methionine in type 2. Type 14 which also has isoleucine at position 153 has an asparagine at position 380 rather than an aspartic acid. These variations may also be included among other

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substitutions in the nucleic acid and amino acid compositions of this invention which provides modified pneumolysin in which at least one epitope is preserved.

Modified pneumolysin polypeptides are provided by this invention which have reduced or no hemolytic activity compared to the wild-type and retain a sufficient number of epitopes to produce antibodies cross reactive with native or wild-type pneumolysin. Identification of such polypeptides is accomplished by first inserting random mutations into the gene encoding pneumolysin and then screening the expressed polypeptide products for loss or reduction of activity associated with toxicity.

#### 1. Methods Of Modifying Pneumolysin

A novel screening system useful for making and identifying substantially immunogenic, but non-toxic or minimally toxic pneumolysins useful in immunizing against *S. pneumoniae* infections is provided by this invention.

This method comprises two basic steps: (1) random mutagenesis and (2) selection.

Random mutagenesis is one of the suitable techniques for introducing mutations into pneumolysin. Standard mutagenesis methods are suitable for use with this invention. In an embodiment, random PCR is performed in order to randomly incorporate nucleotide changes into the type 14 pneumolysin genome. The subsequent selection will identify desirable changes. This method is applicable with any isolated pneumolysin gene. Preferably, enough of the nucleic acid sequences is identified to enable production of oligonucleotide probes.

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Non-limiting examples of such pneumolysin genes are those encoding for type 2 and 14 pneumolysin. The nucleotide sequence encoding type 14 is shown in Fig. 1.

5       PCR, or nucleic acid amplification, is described  
in U.S. Patent Nos. 4,183,195, 4,965,188 and 5,176,995,  
which are incorporated herein by reference. Generally,  
PCR is a method for amplifying one or more specific  
nucleic acid sequences wherein each sequence consists of  
two separate complementary strands. PCR requires  
10    hybridizing each strand with a complementary  
oligonucleotide primer. These nucleic acids are templates  
for synthesis of complementary strands using primers as  
described below. An extension product of each primer is  
then synthesized which is complementary to each nucleic  
15    acid strand. Next, the extension products are separated  
from the templates on which they were synthesized to  
produce single stranded molecules. Finally, the single  
stranded molecules are again treated with the primers of  
the first step under conditions such that an extension  
20    product is synthesized for each of the single stranded  
molecules produced in the second step. These steps may be  
repeated for optimal amplification of the original nucleic  
acid and product synthesis.

PCR mutagenesis involves incorporation of a  
25    "mismatch" nucleotide into the growing strand and may be  
facilitated by reliance on the high error rate of commonly  
used PCR polymerases. Other methods, known in the art for  
creating random mutations may also be used such as, for  
example chemical mutagenesis (Eichenleub, R. (1979) *J.*  
30    *Bacteriol.* **138**:559-566.) Alternatively, the mutagenesis



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step may be accomplished by PCR using a "semi-random" process in which either one or both primers include a random series of nucleotides but a portion of one or both primers is complementary and thus "anchored" to at least one known pneumolysin sequences.

"Primers," as that term is used herein, refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of nucleic acid synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e. in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH. The primers are preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare amplification products. Preferably, the primers are oligodeoxyribonucleotides but must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and use of the method. The primers typically contain 10 or more nucleotides.

Synthetic oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods (Narang, S.A. et al. (1979) *Meth. Enzymol.* **68**:90; Brown E.L., et al. (1979) *Meth. Enzymol.* **68**:109) or automated embodiments

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thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucauge et al. (1981) *Tetrahedron Let.* **22**:1859-1962. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066 which is incorporated herein by reference.

It is also possible to use a primer which has been isolated from a biological source. One such example may be a restriction endonuclease digest of a large nucleic acid molecule encoding pneumolysin which is sufficiently complementary to hybridize to the pneumolysin sequences. Nucleotide substitutions may also be inserted into primers during chemical synthesis.

It is to be understood that the nucleotide sequences of this invention need not be limited to a single mutation within any given molecule encoding the modified pneumolysin polypeptides. Multiple mutations are also possible when they preserve the immunogenic character of native pneumolysin polypeptide (see Fig. 2), while attenuating or eliminating one or more of its toxic characteristics. Multiple modifications may therefore be included in a single polypeptide molecule (see Fig. 4). Multiple modifications may be useful because they may reduce the likelihood of reversion to the toxic native sequence. However, a preferred embodiment of this invention is single mutations in the nucleic acid sequence which result in single amino acid substitutions.

The random or semi-random PCR products encoding modified pneumolysin, may be cloned into an appropriate

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expression vector using standard cloning techniques known in the art.

In an embodiment, the vector includes at least one possible cloning site, at least one antibiotic  
5 selection marker gene, transcription promoter and an origin of replication. The vector may be grown in a variety of compatible host cells, allowing a high degree of expression. Preferred hosts include bacteria such as *E. coli*, *B. subtilis* or yeast such as *S. cerevisiae*.  
10 Other eukaryotic cells besides yeast such as mammalian cells may also be used, for example. The cloning plasmid vector/host cell combination may be any compatible vector and host cell. Any suitable expression vector and host cell are acceptable provided they are able to support the  
15 expression of the modified pneumolysin. Standard protocols for cloning and expression may be used as described in Ausubel, F.M. et al., eds. (1997) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. which is incorporated herein by reference.

## 20                                   2.     **Screening Of Modified Pneumolysin**

Following ligation of the modified pneumolysin nucleotide sequence to the vector in proper reading frame and transformation into the host cell, screening is  
25 performed in order to identify cell clones expressing modified pneumolysin polypeptides which have reduced or absent toxicity.

A method for identifying suitably transformed hosts expressing the randomly mutated pneumolysin polypeptide is provided by this invention. Preferred

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modified pneumolysin polypeptides will have similar structural features such as size when compared to native pneumolysin. Therefore selection methods which analyze polypeptide size such as SDS-PAGE and gel permeation

5 chromatography maybe used. Transformed hosts expressing modified pneumolysin maybe identified by analyzing the proteins expressed by the host using SDS-PAGE and comparing the gel to an SDS-PAGE gel obtained from the host which was transformed with the same vector but not  
10 containing a nucleic acid sequence coding for pneumolysin or modified pneumolysin (the "standard host").

Transformed hosts expressing pneumolysoid will produce a new band when examined by SDS-PAGE and transformed hosts producing a large band corresponding to pneumolysoid can  
15 be selected as candidates. The modified pneumolysin polypeptides expressed by these clones may then be

screened for hemolytic activity in the cell extracts to identify the modified pneumolysin polypeptides that have attenuated hemolytic activity. Transformed hosts

20 producing non-modified or modified yet active pneumolysin which are toxic can be eliminated by this simple screening step.

Alternatively, modified pneumolysin can be identified by other methods known to those of ordinary  
25 skill in the art such as, but not limited to, SDS-PAGE, followed by electroblotting or western blotting analysis, or dot blotting of total cell extracts, or limited proteolysis of the soluble fraction and further analysis of the digests by SDS-PAGE or western blotting.

30 Factors to be considered in choosing the method

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of pneumolysin purification and isolation include whether the modified pneumolysin is present as a soluble protein or whether it becomes insolubilized in inclusion bodies. Although not a general rule, mutations which affect the folding properties of pneumolysin appear to favor its accumulation in inclusion bodies.

Modified pneumolysin which has been identified in the soluble fraction of the cell extracts may be isolated and purified by conventional methods of purification, such as, but not limited to: precipitation of nucleic acids, salt fractionation or capture procedures such as ion exchange chromatography or hydrophobic interaction chromatography. Gel permeation chromatography may be used, particularly as a polishing step, following one of the aforementioned chromatographic procedures. Alternatively, the recombinant modified pneumolysin may be isolated by affinity chromatography, or by procedures used for isolation of thiol-containing proteins, as well as other methods known to those of ordinary skill in the art (Current Protocols in Protein Science, 1995 John Wiley & Sons).

Alternatively, modified pneumolysin derived from the inclusion bodies may be isolated following several inclusion body washes to remove nucleic acids and other bacterial cell wall contaminants. This procedure may include, but is not limited to, washing the pellet with regular buffers, or regular buffers and detergent additives. The protein may be further purified under denaturing conditions by dissolving the washed inclusion bodies in urea or guanidine HCl followed by gel filtration

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chromatography. This procedure can be done prior to protein refolding. However, refolding followed by ion-exchange chromatography represents a preferred method to achieve maximal yields of refolded and purified protein.

5 Native pneumolysin can be obtained by the procedure described and used as reference. The hemolytic activity and the migratory or elution profile of the native counterpart can thus be used as reference for the isolation of modified pneumolysins from either the soluble  
10 or inclusion body fractions.

Preferred criteria for selecting clones expressing suitable modified pneumolysin polypeptides include one or more of: (1) modified pneumolysin expression; (2) at or near full length expression (based  
15 on a molecular weight of about 53,000 for native pneumolysin); (3) presence of pneumolysoid in the soluble fraction; (4) low hemolytic activity; and (5) high yield of expressed polypeptide.

Although the inclusion of all the above criteria  
20 in a screening protocol would identify the most efficient and likely useful clones expressing a useful modified pneumolysin polypeptide, less efficient clones may also produce modified pneumolysins which are suitable for use in this invention including some that may not be full  
25 length, but are sufficiently long to elicit production of antibodies cross-reactive with native pneumolysin and/or function as carrier polypeptides in a polysaccharide-polypeptide conjugate molecule.

Although the preferred method for identifying  
30 desirable clones described above directly assays

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characteristics of expressed protein including size and hemolytic activity, other methods such as detecting cross-reactivity with antibodies directed against native pneumolysin or hybridization to nucleic acid probes may also be used. In one embodiment, initial identification of host cell clones transformed with plasmids containing the modified pneumolysin nucleic acid sequences may be performed using standard hybridization analysis as known to those skilled in the art. Probes for modified pneumolysin genes include native pneumolysin nucleic acid sequences or the amplification primers or other primers indicating the presence of the amplified sequences. Preferably such hybridizing probes are 30 to 40 nucleotides in length; more preferable 10 to 20 nucleotides in length. Stringency should be relatively low since probes may be hybridizing to sequences containing altered bases.

A preferred method of hybridization is blot hybridization. See Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* 2nd Ed., Cold Spring Harbor Laboratory Press which is incorporated herein by reference, for additional details regarding blot hybridization. A probe can be DNA or RNA and can be made detectable by any of the many labeling techniques readily available and known to the skilled artisan. Such methods include, but are not limited to, radio-labeling, digoxigenin-labeling, and biotin-labeling. A well-known method of labeling DNA is  $^{32}\text{P}$  using DNA polymerase, Klenow enzyme or polynucleotide kinase. In addition, there are known non-radioactive techniques for signal amplification

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including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al. (1973) *Proc. Natl. Acad. Sci. USA* **70**:2238-42), methods which allow detection by chemiluminescence (Barton, S.K. et al. (1992) *J. Am. Chem. Soc.* **114**:8736-40) and methods utilizing biotinylated nucleic acid probes (Johnson, T.K. et al. (1983) *Anal. Biochem.* **133**:125-131; Erickson, P.F. et al. (1982) *J. Immunol. Methods* **51**:241-49; Matthaei, F.S. et al. (1986) *Anal. Biochem.* **157**:123-28) and methods which allow detection by fluorescence using commercially available products. Non-radioactive labeling kits are also commercially available.

The screening process includes testing of the pneumolysoid-expressing cells for low hemolytic activity by methods which are known in the art. (Bernheimer, A. (1988) *Meth. Enzymol.* **165**:213-217.) A micro-assay may be performed in a 96-well, U-bottom, micro-titer plate, using an aliquot of culture grown from colonies positive for pneumolysin (native or modified) expression determined as described above. The aliquots may be extracted and normalized for polypeptide content. The extracts may further be centrifuged and the resulting pellet cell debris and the supernatant analyzed separately. Further identification of pneumolysoid expression in the supernatant indicates availability in the solubilized fraction.

Aliquots of the cell lysates may be obtained, pelleted by centrifugation and the supernatant or pellet analyzed for activity. Screening the pellets for activity involves solubilization with a denaturant, such as urea,



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followed by serial dilutions which are conducted as described for the soluble species. Using this procedure the protein undergoes refolding and activity, if present, can be detected.

5           Negative activity results imply either an inactive refolded polypeptide or an improperly refolded polypeptide. To distinguish between these two conditions, a second screening process can be used. Activity-negative clones are denatured and refolded before loading onto an  
10   ion-exchange chromatography column. The mutants which have an elution pattern similar to wild-type pneumolysin can be further analyzed by gel-filtration chromatography and monomeric species with a Stokes radius similar to wild-type pneumolysin are selected.

15           The inserted nucleic acid sequence encoding the modified pneumolysin of selected clone(s) may be sequenced by any of the methods commonly used in the art and the corresponding amino acid sequences deduced.

## **B.   Modified Pneumolysin Polypeptides**

### **1.   Reduction of hemolytic activity**

20           The modified pneumolysin polypeptides of this invention are polypeptides that are non-hemolytic or substantially non-hemolytic and still maintain at least one epitope that binds to antibody directed against the  
25   native polypeptide. Because such hemolytic activity is associated with the toxicity of pneumolysin, the modified pneumolysins would therefore also be expected to be less toxic than native pneumolysin. The modified pneumolysin

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polypeptides of this invention contain at least one mutation relative to *S. pneumoniae* type 14 wild-type pneumolysin (Fig. 3), preferably among the first 257 amino acids beginning from the N-terminus. Modification of as few as one amino acid is required to result in modified pneumolysin polypeptides which have little or insignificant toxicity as determined by hemolytic assay. Thus, substitutions at any one, or more, of positions 61, 148 and 195 may result in polypeptides having reduced hemolytic activity. Preferred substitutions for amino acids 61, 148 and 195 are shown below in Table 1.

**Table 1**

	Amino Acid Position		
	61	148	195
Wild-type	Ser	Met	Phe
Substitutions	Pro	Lys	Ile/Val

Substitutions at these preferred positions with amino acids other than the preferred ones, for example, those having similar charge at neutral pH, are also within the scope of this invention. Accordingly, substitution of the serine at 61 with hydroxyproline; methionine at 148 with arginine or histidine; phenylalanine at 195 with leucine, glycine or alanine are other non-limiting examples of possible substitutions.

Although single substitutions may be sufficient

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to attenuate hemolytic activity, such reduction may also be accomplished by substituting in a single polypeptide specific groups of amino acids. For example, the collective substitution in a single polypeptide of the amino acids at positions 33, 46, 83, 239 and 257 produces polypeptides having characteristics of pneumolysin but with reduced hemolytic activity. Preferred substitutions are shown in Table 2.

**Table 2**

	Amino Acid Position				
	33	46	83	239	257
Wild-type	Ile	Ile	Leu	Ser	Asp
Substitution	Thr	Thr	Ser	Arg	Gly

As with the single substitution, other amino acids in addition to those which are preferred may also be substituted based on the same considerations of charge discussed above with the further non-limiting example that serine and threonine may be substituted for each other, and that other neutral amino acids such as those recited above may be substituted for Asp at 257.

It should be understood that besides the substitutions disclosed above, which are effective for reducing or eliminating the hemolytic activity, other substitutions may also be made provided that at least one epitope capable of binding an antibody which binds native pneumolysin is retained. Non-limiting examples of amino

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acid residues which may be substituted but which alone do not reduce hemolytic activity include those at positions 17, 18, 33, 41, 45, 46, 63, 66, 83, 101, 102, 127, 128, 172, 189, 239, 255 and 257. Examples of substitutions at these positions include, but are not limited to those shown in Table 3. Because these sites are not associated with decreases in hemolytic activity it is expected that these positions may be more freely substituted with less regard to size and charge.

**Table 3**

		Amino Acid Position								
		17	18	33	41	45	46	63	66	83
Wild-type		Lys	Lys	Ile	Asp	Val	Ile	Thr	Asn	Leu
Substitution		Arg	Asn	Thr	Gly	Ala	Thr	Ser	Tyr	Ser

		Amino Acid Position								
		101	102	127	128	172	189	239	255	257
Wild-type		Ile	Asp	Val	Asn	Thr	Gln	Ser	Lys	Asp
Substitution		Thr	Gly	Glu	His	Ala	Arg	Arg	Gly	Gly

It is to be understood that the amino acid substitutions described above are not exhaustive and that other modified pneumolysin polypeptides identified

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according to the methods of this invention are also within its scope.

Single point mutations of the native pneumolysin sequence are preferred because the antigenic nature of the native pneumolysin polypeptide is more likely to be preserved by the single point modified form. Alternatively, a combination of multiple mutations, may be used.

However, multiple mutations are sometimes unpredictable. The mutations, in some cases, may act synergistically to abolish activity or they may be involved in compensation mechanisms during folding. For these reasons, single point mutations are considered to be advantageous.

Although the screening process is based on identifying modified pneumolysin polypeptides which are substantially full-length, this invention also encompasses fragments and truncated forms of the modified pneumolysin polypeptides provided they retain at least one epitope recognized by an antibody which binds to the mature pneumolysin. In addition, it is preferred that such fragments or truncated forms be of sufficient size to produce polysaccharide-polypeptide conjugates which produce a T cell dependent immune response.

The hemolytic activity of the pneumolysoid proteins of this invention may vary over a wide range depending on how the pneumolysoid is actually used. For example, conjugation of a pneumolysoid with reduced hemolytic activity may reduce such activity further to acceptable levels. Conversely, where a pneumolysoid is to be introduced into an individual, unconjugated to another

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component or where it may be cleaved, it will be desirable to have the hemolytic activity reduced as close to the minimum detectable level as possible. For such purposes, levels of hemolytic activity between about 0.2% and about 0.5%, or more preferably about 0.2% are suitable. Where some hemolytic activity may be tolerated, or where such activity may be further attenuated by, for example, conjugation to polysaccharide, higher levels of hemolytic activity may be acceptable, i.e. from about 0.5% to about 25%, or more preferably between about 1% and about 10%.

## 2. Protein structure

Previous studies report that the C-terminus of PLY contains the cell-binding site (Owen et al., 1994 FEMS Microbiol. Let. 121, 217-221). The mutagenesis studies of this invention were focused on the N-terminus which reportedly contains the oligomerization domain. The finding that pre-incubation of erythrocytes with certain mutants abrogated the wild type hemolytic activity in a concentration dependent manner indicates that these mutants are indeed capable of competing with the wild type counterpart for the cell binding site. Since the mutants inhibit wild type activity, these mutants likely retain the structural features of wild-type pneumolysin. The preservation of the cell binding domain in the mutant forms, specifically in the case of pNV103 and pNV207 is significant as these mutants also exhibit the immunological properties of the wild type molecule, as evidenced in ELISA inhibition assays. Moreover, antibodies generated against these mutants possess the

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ability to neutralize the hemolytic activity of the wild type counterpart, additional evidence of their native-like structure.

5       The structural features and integrity of wild type pneumolysin and selected mutants have also been assessed by circular dichroism and fluorescence spectroscopy. These techniques offer the unique advantage of providing both qualitative and quantitative information on the secondary and tertiary structure of these proteins.

10      Wild type pneumolysin is characterized by a high content of  $\beta$ -sheet structure, a prominent feature in the far UV CD spectra of all the mutants selected in the present study. The shape of the spectra and deconvolution analysis are consistent with previous studies on recombinant

15      pneumolysin purified from soluble fractions of *E. coli* which was structurally and functionally equivalent to the native pneumococcal pneumolysin (Mitchell et al., 1989 Biochem. Biophys. Acta 1007, 67-72). Likewise, both the near UV CD and fluorescence spectra are consistent with

20      the native structure containing Trp residues (Morgan et al., 1993 Biochem. J. 296, 671-674) whose side chains are partially exposed to solvent, as evidenced by the emission maximum at ~345 nm upon excitation at 290 nm. The unique near UV CD spectra characterized by a minimum ellipticity

25      at ~280 and a maximum ellipticity at ~290 nm, represents a fingerprint of this (Morgan et al., 1993) and other cytolysins, such as perfringolysin (Nakamura et al., 1995 Biochemistry 34, 6513-6520). As such, this characteristic spectroscopic fingerprint may represent a useful baseline

30      measurement for subsequent evaluation of batch-to-batch

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consistency, particularly for those mutants selected as components of vaccine candidates.

**C. Nucleic Acid Molecules Encoding Modified Pneumolysin**

5           The modified pneumolysin polypeptides of this invention are preferably synthesized by expressing a nucleic acid molecule encoding the modified polypeptide in a host microorganism transformed with the nucleic acid molecule. Accordingly, this invention also encompasses  
10 the nucleic acid molecules, including DNA and RNA encoding the modified pneumolysins discussed above.

          The DNA encoding the polypeptides of the invention may be used to express recombinant polypeptide in a wide variety of host cells using a wide variety of  
15 vectors. The host cell may be prokaryotic or eukaryotic. DNA for native wild-type pneumolysin may be obtained from natural sources, such as *Streptococcus pneumoniae*, or alternatively synthesized. The wild-type DNA may then be used as the starting material for modification, as  
20 described above, to obtain the DNA encoding the modified pneumolysin polypeptides of this invention. Once identified as encoding desirable modified pneumolysin polypeptides, the DNA encoding such polypeptides may then be cloned into various vectors for expression.  
25 Alternatively, the genes encoding such polypeptides may also be synthesized in whole or in part.

          In one embodiment, the invention relates to a method of expressing the modified pneumolysin polypeptide in a microorganism wherein the microorganism is



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transformed by a vector comprising a gene encoding the modified pneumolysin polypeptide wherein the polypeptide so produced comprises more than about 2% of the total protein expressed in the transformed microorganism. In yet another embodiment, the modified pneumolysin polypeptide expressed comprises more than about 40% of the total proteins expressed in *E. coli*.

Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Non-limiting examples of some suitable prokaryotic vectors include plasmids from *E. coli*, such as *colE1*, *pCR1*, *pBR322*, *pMB9*, and *RP4*. Prokaryotic vectors also include derivatives of phage DNA such as *M13*, *fd*, and other filamentous single-stranded DNA phages.

The modified pneumolysin polypeptides can be expressed either direct or as fusion constructs. Two non-limiting examples of fusion constructs are Thiofusion and His-Tag which can be isolated and purified by conventional methods. Vectors for expressing proteins in bacteria, especially *E. coli*, are also known. Such vectors include, but are not limited to, *pK233* (or any of the *tac* family of plasmids), *pT7*, and *lambda pSKF*. Examples of vectors that express fusion proteins include the *PATH* vectors described by Dieckmann and Tzagoloff (1985) in *J. Biol. Chem.*

**260:**1513-1520. These vectors contain DNA sequences that encode anthranilate synthesis (*TrpE*) followed by a polylinker at the carboxy terminus. Two non-limiting examples of fusion constructs are Thiofusion and His-Tag which can be isolated and purified by conventional methods. Other expression vector systems are based on

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beta-galactosidase (pEX); maltose binding protein (pMAL); and glutathione S-transferase (pGST) -(see (1988) *Gene* **67**:31 and (1990) *Peptide Research* **3**:167). See Ausubel et al., *supra*.

5                    Vectors useful in yeast are also available. Suitable examples are YIp, YRp, YCP, YEp and YLp plasmids. See Ausubel, Id.

                  Suitable vectors for use in mammalian cells are also known. Such vectors include well-known derivatives  
10 of SV-40, adenovirus, retrovirus-derived DNA sequences and vectors derived from combination of plasmids and phage DNA. Additional vectors for eukaryotic expression vectors are reported in (e.g., P.J. Southern and P. Berg (1982) *J. Mol. Appln. Genet.* **1**:327-341; S. Subramani et al. (1981)  
15 *Mol. Cell. Biol.* **1**:854-864; R.J. Kaufmann and P.A. Sharp (1982) *J. Mol. Biol.* **159**:601-621; R.J. Kaufmann and P.A. Sharp (1982) *Mol. Cell. Biol.* **159**:601-664; S.I. Scahill et al. (1983) *Proc. Natl. Acad. Sci. USA* **80**:4654-4659; G. Urlaub and L.A. Chasin (1980) *Proc. Natl. Acad. Sci. USA*  
20 **77**:4216-4220.

                  Examples of preferred vectors are plasmids, and some non-limiting examples of plasmids containing the T7 inducible promotor, include the expression plasmids pET-17b, pET-11a, pET-24a-d(+) and pET-9a, all of which are  
25 commercially available from Novagen (565 Science Drive, Madison, Wis. 53711). These plasmids comprise, operatively linked, in sequence, a T7 promoter, optionally a lac operator, a ribosome binding site, restriction sites to allow insertion of the structural gene and a T7  
30 terminator sequence. See, Novagen catalogue (1993) at 36-

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43.

Useful expression hosts include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* BL21 (DE 3), *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DHI, and *E. coli* MRC1, *Pseudomonas*, and *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeasts such as *Saccharomyces* and other fungi, insect, animal cells, such as COS and CHO cells, human cells and plant cells in tissue culture.

In a preferred embodiment, *E. coli* strain BL21 (DE3) is employed. The above mentioned plasmids may be transformed into this strain.

Selection of *E. coli* transformed with the desired vectors may be accomplished using standard selection protocols involving growth in a selection medium which is toxic to non-transformed cells. For example, *E. coli* is grown in a medium containing a selection agent, e.g. any  $\beta$ -lactam to which *E. coli* is sensitive such as ampicillin. The pET expression vectors provide selectable markers which confer antibiotic resistance to the transformed organism.

High level expression of the modified pneumolysin polypeptide can be toxic in *E. coli*. Surprisingly, this invention allows for selection of modified pneumolysin polypeptides which may be expressed in *E. coli* to a level of at least about 40% of total cellular proteins.

Additional nucleotide mutations may be made that

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were not identified in the selection process particularly where the translated amino acid is the same as the identified amino acid predicted based on the sequence of the selected clone. In addition, nucleotide changes may be made which encode conservative amino acid substitutions, especially where the identified polypeptides exhibit other amino acid substitutions. Conservative amino acid substitutions are known in the art and represent substitutions of "similar" amino acids. Considerations include, but are not limited to polarity, hydrophobicity, size, and side chain structure.

The modified pneumolysin polypeptides of this invention are polypeptides that are non-toxic or substantially non-toxic and still retain at least one epitope that binds antibody directed at native pneumolysin. The modified pneumolysin of this invention contain at least one mutation relative to wild-type pneumolysin, preferably among the first 257 amino acids of the N-terminus. The modified pneumolysin may be altered in that the amino acid present at one, or more than one, of residue sites 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 127, 128, 148, 172, 189, 195, 239, 243, 255, 257, 286 or 446 of wild-type pneumolysin are replaced, removed or blocked. As discussed above, additional modifications can be incorporated from other known, modified pneumolysin polypeptides such as those at residue sites 367, 379, 384, 385, 397, 428, 433, 434 or 435 which are disclosed in PCT WO 90/06951 which is incorporated herein by reference. In addition to the amino acid substitutions of this invention disclosed herein, other

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amino acid substitutions that have been reported (Hill et al. (1994) *Infection and Immunity* 62, 757-758) for pneumolysin may also be used with this invention provided they allow for refolding of the pneumolysin as determined by the methods described herein. Hill et al. reports four N-terminal region mutations, Arg-31→Cys, Leu-75→Phe, Val-127→Gly and His-156→Tyr that result in 75%, 100%, 75% and 2% hemolytic activity, respectively. They also report four C-terminal region mutations, Ala-432→Val, Trp-433→Arg, Trp-436→Arg and Val-468→Leu that result in 100%, <1%, 50% and 100% hemolytic activity, respectively. However, if any of these mutations result in improperly refolded pneumolysoids, then it is preferred they not be used. The preferred modifications of pneumolysin are those at residue sites 61, 148 or 195 and the most preferred is at residue 195. Additionally, the combination of modifications at sites 33, 46, 83, 239 and 257 is also preferred.

Specific changes may be introduced into the native pneumolysin sequence by any of the methods for site-directed mutagenesis known in the art. In a preferred embodiment, PCR may be performed using oligonucleotide amplification primers encoding the desired nucleotide substitution(s) within their sequence.

Alternatively, the modified pneumolysoid polypeptide may be constructed by chemical synthesis. (Kent et al. *Adv. Exp. Med. Biol.*, 1995, 362, 425-438). Such synthesis can be used to make all or part of a pneumolysoid. In the case of partial synthesis, the

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synthetic peptide can be covalently bound to an appropriate portion of the pneumolysoid peptide, prepared by methods known in the art or taught herein, to generate a semi-synthetic pneumolysoid.

5                   **D.    Vaccine and Antibody Preparations**

                  This invention is also directed to vaccine and antibody preparations. According to this invention, the expressed, modified pneumolysin described above or its derivatives or fragments thereof may be used as an  
10                   immunogen to generate antibodies that are reactive against pneumolysin.

**1.    Antibodies**

                  The recombinant techniques for polypeptide expression described above, provide for the production of  
15                   abundant amounts of the modified pneumolysin polypeptides of this invention, based on the nucleic acid sequences of this invention. This facilitates the generation of antibodies reactive against the modified pneumolysin polypeptide. However, it should be understood that the  
20                   polypeptide may also be synthesized by chemical methods or combinations thereof.

                  In another embodiment, antibodies directed against the modified pneumolysin polypeptides may be generated by any of the techniques that are well known in  
25                   the art. According to one approach, the antibodies may be generated by injecting an isolated modified pneumolysin polypeptide preparation or derivatives or fragments

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thereof into a host animal. The host animal may be, but is not limited to, rat, mouse, rabbit, non-human primate, or a human. Immunological responses may be increased by the use of adjuvants which are known in the art.

5           Monoclonal antibodies directed against the modified pneumolysin polypeptide may also be prepared by any of the techniques that are well known in the art. According to one method, cultures of continuous hybridoma cell lines are used (Kohler and Milstein (1975) *Nature*  
10   **256**:495-497). Monoclonal antibodies directed against the modified pneumolysin polypeptide may be human monoclonal antibodies or chimeric monoclonal antibodies made by any of the techniques that are well known in the art. According to one approach, chimeric monoclonal antibodies  
15 may be generated that have a non-human (e.g. mouse) antigen-binding domain combined with a human constant region. (Takeda et al. (1985) *Nature* **314**:452).

          Antibodies directed against the modified pneumolysin polypeptide may be purified by any of the  
20 techniques that are well known in the art including, but not limited to immunoabsorption or immunoaffinity chromatography, or other chromatographic methods (e.g. HPLC, gel filtration or ion exchange). Antibodies may also be purified as immunoglobulin fractions from serum,  
25 plasma or cell culture medium.

          Antibody molecules of this invention may be intact immunoglobulin molecules, substantially intact immunoglobulin molecules, or those portions of an immunoglobulin molecule, for example Fab fragments, that  
30 contain the antigen binding site.

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Fragments of antibodies directed against the modified pneumolysin polypeptide may be generated by any of the techniques that are well known in the art.

(Campbell (1985) *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13, Burdon, et al. (eds.), Elsevier Science Publishers, Amsterdam).

## 2. Conjugate Molecules

The modified pneumolysin polypeptides of this invention may be used to elicit an antibody response to *S. pneumoniae* in an individual either alone or when conjugated to another immunogenic molecule such as a polysaccharide. The other immunogenic molecule, may be derived from either *S. pneumoniae*, or from a different infectious agent against which it is desirable to generate an immune response. Preferably the other immunogenic molecule to which the modified pneumolysin is conjugated is a capsular or non-capsular polysaccharide from a pathogenic bacteria. Such bacteria including for example: *Haemophilus influenzae* type b; meningococcus group A, B, or C; group B or A streptococcus of various serotypes including group B types Ia, Ib, II, III, V, and VIII; as well as the various serotypes of *S. pneumoniae* preferably types 1-23. *S. pneumoniae* serotypes 3, 4, 6b, 9v, 14, 18c, 19f and 23 are most preferred. Such polysaccharides for use to conjugate pneumolysoid may also be modified themselves in order to be more effective or reduce cross-reactivity to endogenous epitopes. See, for example, Jennings et al. U.S. patents 4,727,136, 5,576,002 and U.S application serial number 08/484,569 which is published as international application



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WO 96/40239 which are incorporated herein by reference for modification to group B meningococcal polysaccharides.

Any mode of conjugation may be employed to conjugate the polysaccharide components with the modified pneumolysin polypeptide. A preferred method is that described in U.S. Patent No. 4,356,170, i.e. by introducing terminal aldehyde groups (via oxidation of cis-vicinal hydroxyl groups) into the polysaccharide and coupling the aldehyde groups to the polypeptide amino groups by reductive amination. The polysaccharide and the modified pneumolysin polypeptides are thereby linked through a  $-CH_2-NH-$ polypeptide linkage.

It is to be understood, however, that the conjugate vaccines of the invention are not limited to those produced via reductive amination. Thus, the vaccines may also be produced by conjugating the polysaccharide with the modified pneumolysin polypeptide using an adipic dihydrazide spacer, as described by Schneerson, R., et al. (1980) *J. Exp. Med.* **1952**:361-476, and in U.S. Patent No. 4,644,059. Alternatively, the binary spacer technology developed by Merck may be used, as described by Marburg, S. et al. (1986) *J. Am. Chem. Soc.* **108**:5282-5287 or, possibly, the reducing ends methodology.

Conjugate molecules prepared according to this invention typically comprise at least one modified pneumolysin polypeptide of the present invention to which is bound at least one polysaccharide component. Thus, this invention provides the ability to produce conjugate molecules wherein the polypeptide is linked to the

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polysaccharide through at least two sites to create cross-linked conjugates.

The vaccines of this invention may provide active or passive immunity. Vaccines for providing active immunity comprise a purified modified pneumolysin polypeptide of this invention. Preferably the polypeptide of this vaccine comprises at least one of the following amino acid substitutions in the wild-type pneumolysin amino acid sequence as shown in Table 1.

In another embodiment of this invention, antibodies directed against the modified pneumolysin polypeptide of this invention may be used as a pharmaceutical preparation in a therapeutic or prophylactic application in order to confer immunity from a host individual to another individual (i.e. to augment an individual's immune response against *S. pneumoniae* or to provide a response in immuno-compromised or immuno-depleted individuals including AIDS patients). Passive transfer of antibodies is known in the art and may be accomplished by any of the known methods. According to one method, antibodies directed against the modified pneumolysin polypeptides or conjugates thereof of this invention are generated in an immunocompetent host ("donor") animal, harvested from the host animal and transfused into a recipient individual. For example, a human donor may be used to generate antibodies reactive against the modified pneumolysin polypeptide or conjugate of this invention, the antibodies transfused in therapeutically or prophylactically effective amounts into a human recipient in need of treatment, thereby conferring

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resistance in the recipient against not only the pneumolysin toxin, but against *S. pneumoniae* and a bacteria which bind antibodies elicited by the polysaccharide component if the donor was immunized with a conjugate.

### **E. Pharmaceutical Compositions**

The pharmaceutical compositions of this invention may comprise the modified pneumolysin polypeptides, conjugate molecules comprising the modified polypeptides or compositions comprising antibodies elicited by one of the modified pneumolysin polypeptide compositions of this invention. These pharmaceutical compositions are particularly useful as vaccines.

For eliciting passive immunity, the pharmaceutical composition may be comprised of, polyclonal antibodies or monoclonal antibodies or their derivatives or fragments thereof as described above. The amount of antibody, fragment, or derivative will be a therapeutically or prophylactically effective amount as determined by standard clinical techniques.

The pharmaceutical preparations of this invention may be introduced to an individual by methods known to be effective in the art. Intradermal, intraperitoneal, intravenous, subcutaneous, intramuscular, oral, and intranasal are among, but not the only routes of introduction.

The compositions of the invention may comprise standard carriers, buffers or preservatives known to those in the art which are suitable for vaccines including, but

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not limited to any suitable pharmaceutically acceptable carrier, such as physiological saline or other injectable liquids. Additives customary in vaccines may also be present, for example stabilizers such as lactose or sorbitol, and adjuvants to enhance the immunogenic response such as aluminum phosphate, hydroxide, or sulphate and stearyl tyrosine. The vaccines produced according to this invention may also be used as components of multivalent vaccines which elicit an immune response against a plurality of infectious agents.

Vaccines of the present invention are administered in amounts sufficient to elicit production of antibodies as part of an immunogenic response. Dosages may be adjusted based on the size, weight or age of the individual receiving the vaccine. The antibody response in an individual can be monitored by assaying for antibody titer or bactericidal activity and boosted if necessary to enhance the response. Typically, a single dose is about 0.1 to 10 µg/kg.

#### **F. Diagnostic Kits**

In another preferred embodiment, the modified pneumolysin polypeptides of this invention or derivatives or fragments thereof may be used to produce safer diagnostic kits that do not incorporate pneumolysin toxin but can still indicate the presence of antibodies directed against *S. pneumoniae*. The presence of such antibodies can indicate prior exposure to the pathogen, and predict individuals who may be resistant to infection. An antibody reaction may be identified by any of the methods

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described in the art, including but not limited to an ELISA assay. Such knowledge is important, and can avoid unnecessary vaccination. The diagnostic kit may comprise at least one of the modified pneumolysin polypeptides of this invention or derivatives or fragments thereof and suitable reagents for the detection of an antibody reaction when the modified polypeptides or derivatives or fragments are mixed with a sample that contains antibody directed against pneumolysin.

Alternatively, the diagnostic kit may further comprise a solid support or magnetic bead or plastic matrix and at least one of the modified pneumolysin polypeptides of this invention or derivatives or fragments thereof.

In some cases, it may be preferred that the polypeptides or derivatives or fragments are labeled. Labeling agents are well-known in the art. For example, labeling agents include but are not limited to radioactivity, chemiluminescence, bioluminescence, luminescence, or other identifying "tags" for convenient analysis. Body fluids or tissues samples (e.g. blood, serum, saliva) may be collected and purified and applied to the diagnostic kit. The pneumolysin polypeptides, derivatives (pneumolysoid) or fragments may be purified or non-purified and may be composed of a cocktail of molecules. Antibodies within the sample may or may not react with the pneumolysin.

Solid matrices are known in the art and are available, and include, but are not limited to polystyrene, polyethylene, polypropylene, polycarbonate,

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or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks, plates or the like. Additionally matrices include, but are not limited to membranes, 96-well micro titer plates, test tubes and Eppendorf tubes. In general such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

All publications, patents and articles referred to within the specification are herewith incorporated *in toto*, by reference into the application. The following examples are presented to illustrate the present invention but are in no way to be construed as limitations on the scope of the invention. One skilled in the art will readily recognize other permutations within the purview of the invention.

## EXAMPLES

### Materials and Methods

**Bacterial Strains and Plasmids.** *Streptococcus pneumoniae* serotype 14 (ATCC, Rockville, MD) was used in this study for isolation of genomic DNA. *E. coli* strain DH5 $\alpha$  (Life Technologies, Gaithersburg, MD) was used for initial cloning and production of plasmid DNA. *E. coli* strain BL21 (DE3) $\Delta$ ompA, used for protein expression, was derived from BL21 (BE3) (Novagen) (see U.S. Patent No. 5,439,808 for details). *S. pneumoniae* was grown overnight in Todd-Hewitt (TH) broth at 37°C without shaking under 7.5% CO<sub>2</sub>. *E. coli* strains were grown in Luria-Bertani (LB) broth, supplemented with carbenicillin (50-100  $\mu$ g/ml)

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or kanamycin (50 µg/ml) as needed. The plasmid vectors pUC-19 and/or pBluescript II SK+ (Stratagene) were used for cloning fragments to be sequenced and the plasmids pET-17b and pET-24a (Novagen) were used for cloning  
5 fragments to be expressed.

**SDS-PAGE.** Protein samples were prepared as follows: 1.5 ml fractions were collected from cultures and the cells harvested by centrifugation. The cells were resuspended in 150 µl of protein loading buffer and boiled  
10 for 5 min to lyse the cells. Cell debris were removed by centrifugation and 10 µl of each supernatant were electrophoresed through an 8-16% gradient Tris-glycine "Laemmli" polyacrylamide gel (Novex) along with low molecular weight standards (Bio-Rad). Alternatively,  
15 crude extracts prepared for analysis of hemolytic activity were diluted 1:1 with protein loading buffer and 10-15 µl loaded onto the gel. The protein bands were visualized with Coomassie blue staining.

#### EXAMPLE 1

##### 20 **Expression of pneumolysin.**

*E. coli* strain BL21 (DE3) Δ<sub>ompA</sub> transformed with pET-17b or pET-24a containing the desired gene was grown with moderate aeration at 30°C in LB supplemented with 0.4% glucose and 100 µg/ml of carbenicillin (for pET-17b  
25 constructs) or 50 µg/ml of kanamycin (for pET-24a constructs). When the OD<sub>600</sub> reached 0.6, IPTG was added to a final concentration of 0.4 mM (for pET-17b constructs) of 1 mM (for pET-24a constructs) and the cells were

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allowed to incubate for another 2 h for screening, or 5 h for larger scale production. To assay for pneumolysin levels, 1.5 ml aliquots were removed prior to induction and at various time points after induction and examined by SDS-PAGE.

## EXAMPLE 2

### **Cloning of the pneumolysin gene for *Streptococcus pneumoniae* serotype 14.**

Genomic DNA was isolated from approximately 0.5 g *Streptococcus pneumoniae* serotype 14 using the method described above. This DNA served as the template for two pneumolysin-specific oligonucleotides in a standard PCR reaction. These oligonucleotides were designed to be complementary to the 5' and 3' flanking regions of the pneumolysin gene from *S. pneumoniae* serotype 2 and to contain XbaI restriction sites to facilitate the cloning of the fragment if desired. The sequence of the forward oligonucleotide was 5' AAC CTT GAT TGA TCT AGA TAA GGT ATT TAT GTT GG 3' and the reverse oligonucleotide had the sequence 5' TCT TTT TGT CTC TAG AAT TCT CCT CTC CTA GTC 3'. The PCR reaction conditions were as follows: 200 ng *S. pneumoniae* type 14 genomic DNA, the two oligonucleotide primers described above at 1  $\mu$ M of each, 200  $\mu$ M of each dNTP, PCR reaction buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl<sub>3</sub>, and 2.5 units of Taq polymerase, and QS. to 100  $\mu$ l with dH<sub>2</sub>O. This reaction mixture was then subjected to 25 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 1.5 min. At



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the end of the cycling period, the reaction mixture was loaded on a 1.0% agarose gel and the material was electrophoresed for 2 h after which the band at 1.7 kb was removed and the DNA recovered using GeneClean® (Bio 101).

5 This DNA was then digested with XbaI, repurified and ligated to XbaI-digested pUC-19 using T4 DNA ligase. The ligation mixture was used to transform competent *E. coli* DH5α. Recombinant plasmids were identified and sequenced; many were found to have a DNA sequence consistent with  
10 that of the gene encoding pneumolysin.

### EXAMPLE 3

#### **Expression of the pneumolysin gene in *E. coli*.**

Plasmids capable of expressing the mature pneumolysin protein were constructed by amplifying DNA containing the  
15 full-length pneumolysin gene (pST20, pST85, or type 14 genomic DNA) with nested oligonucleotides designed to isolate the pneumolysin coding region. The forward oligonucleotide was designed to contain a NdeI site and would install a start codon at the 5' end of the coding  
20 region. This primer had the sequence 5' TAT TAG GAG GAG CAT ATG GCA AAT AAA GCA GTA AAT G 3'. The reverse oligonucleotide was designed to contain an XhoI site and had the sequence 5' GGC CTC TTT TTG TCT CGA GCA TTC TCC  
25 TCT CCT AGT C 3'. This strategy allowed the cloning of the fragment encoding mature pneumolysin into the NdeI and XhoI sites of either the pET-17b or pET-24a. Standard PCR was conducted using a template containing the entire pneumolysin gene (type 1, 2 & 14) and the two

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oligonucleotides described above. This PCR reaction yielded a 1.6 kb product when analyzed on a 1.0% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the restriction enzyme NdeI and XhoI. The 1.6 kb product was again gel purified and ligated to NdeI- and XhoI- digested pET-17b or pET-24a using T4 DNA ligase. This ligation mixture was then used to transform competent *E. coli* DH5 $\alpha$ . Colonies that contained the 1.6 kb insert were chosen for further analysis. The DNA from the DH5 $\alpha$  clones was analyzed by restriction mapping and the cloning junctions of the chosen plasmids were sequenced. After this analysis, the DNA obtained from the DH5 $\alpha$  clones was used to transform *E. coli* BL21 (DE3) $\Delta$ ompA. The transformed bacteria were selected on LB-agar containing 100  $\mu$ g/ml of carbenicillin, or 50  $\mu$ g/ml of kanamycin when using the pET-24a plasmid. Typically, several clones were screened for their ability to produce the mature pneumolysin protein.

#### EXAMPLE 4

##### Random Mutagenesis To Generate Modified Pneumolysin.

A portion of the gene encoding pneumolysin comprising amino acid residues 1-257 was subjected to random mutagenesis using a modification of the technique as described. (Cadwell, R.C. and Joyce, G.F. (1994) *PCR Methods Appl.* **3**:pS136-40; Cadwell, R.C. and Joyce, G.F. (1992) *PCR Methods Appl.* **2**:28-33). An oligonucleotide complementary to the T7 promoter region of the pET-24a plasmid (See, Figure 1a) with the sequence 5'ATT ACG CGA CTC ACT ATA GGG 3' and an oligonucleotide complementary to

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a region of the pneumolysin gene around 1250 bp (See Figure 1) with the sequence 5'ATT ACG AAC ATT CCC TTT AGG3' were used to define the region of the gene to be mutated. The random mutagenesis PCR reaction conditions were as follows: purified plasmid pNV-19.2 (100ng), the two oligonucleotide primers described above at 1  $\mu$ M of each imbalance dNTP concentrations of 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, and 1 mM dTTP, PCR reaction buffer (19 mM Tris-HCl, 50 mM KCl, pH 8.3), 8.0 mM MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub>, 6 units Taq polymerase, and QS to 100  $\mu$ l with dH<sub>2</sub>O. This reaction mixture was then subjected to 40 cycles of 95°C for 1 minute, 40°C for 2 minutes, and 72°C for 3 minutes. After the PCR reaction, fragments were extracted with phenol/chloroform and ethanol precipitated. The fragment was then digested with NdeI and HindIII, gel purified and ligated to pNV-19.2, digested with the same enzymes. The fragments were ligated and subsequently transformed into competent BL21 (DE3) *E.coli*.

#### EXAMPLE 5

##### **Selection of Modified Pneumolysin Expressing Modified Pneumolysin Devoid of Toxic Effects.**

The transformation described by Example 4 resulted in numerous colonies (approximately 10<sup>4</sup>) of which 400 were selected randomly for evaluation. The novel screening method described in this example was used to identify colonies that expressed modified pneumolysin polypeptides with the following characteristics: 1) no hemolytic activity, 2) substantially full-length, 3) partially soluble, and 4) monomeric and refoldable when

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isolated from inclusion bodies. This screening method involved the following steps:

(a) testing for presence of low hemolytic activity:

5           A micro-hemolytic assay was used to evaluate the clones. Hemolytic activity-assays were conducted in U-bottom micro titer plates using TBS (Tris-buffered saline, pH 7.4) as an incubation buffer. Following a pre-incubation period of 5 min with 1 mM DTT, twofold serial  
10 dilutions were performed and the samples incubated with an identical volume of a 1% suspension of washed sheep erythrocytes (Cappel) resuspended in the same buffer. The reactions were conducted at room temperature as a function of time (kinetic study), and the extent of erythrocyte  
15 lysis was monitored by visual inspection. Each clone undergoing evaluation was scored from 0-5. A rank of zero indicated no hemolytic activity while a rank of 4-5 indicated hemolytic activity at wild-type levels or above. Two hundred clones with a score of 0,1,2, were selected  
20 and screened again for other desired properties.

(b) testing for expression of full-length pneumolysin polypeptide:

          The polypeptide expression assay was carried out in a 96-well format. Colonies with low hemolytic activity  
25 were evaluated by SDS-PAGE for the presence of a strong band having a molecular weight of about 53,000 Daltons. Full-length pneumolysin has a molecular weight of about 53 kD. Fifty-eight out of 200 were found positive in this assay. These clones were collected for further selection.

30           (c) testing for expression of modified

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pneumolysin polypeptides in the soluble fractions:

Modified pneumolysin polypeptides expressed in both the soluble fraction and inclusion bodies are more likely to be refoldable. Ten ml cultures from 2h IPTG-induced *E. coli* cells harboring plasmids containing mutant pneumolysin sequences lacking or exhibiting reduced hemolytic activity were harvested and resuspended in 1.5 ml of TEN buffer; the cells are lysed by a sequential freezing/thawing/sonication procedure until the supernatant exhibits significant levels of protein, as indicated by the Bradford protein assay, which is indicative of successful lysis. The lysed cell suspension is centrifuged (14,000 rpm/10 min) and aliquots of both, the pellet and supernatant are analyzed by SDS-PAGE. An aliquot of the soluble fraction is tested for hemolytic activity and the hemolytic titer is determined to confirm the reduced activity observed in the kinetic qualitative study conducted in the initial phase of screening. Clones were found that contained soluble, modified pneumolysin polypeptides that had little hemolytic activity.

(d) High yields of refoldable and monomeric, modified pneumolysin polypeptides:

Clones containing soluble pneumolysin are selected for the next step in the screening procedure, which consists of discarding the supernatant by aspiration, washing the pellet with TEN buffer twice, and solubilizing the pellet in 5 ml of 8 M urea prepared in TEN buffer. After sonicating for 2 min, the urea solution is quickly centrifuged to remove aggregates and added dropwise to 45 ml of refolding solution, under constant

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stirring at 4 °C. The refolding solution is then loaded onto a 2 ml DEAE-Sepharose-FF column, pre-equilibrated in Buffer A (25 mM Tris.HCl, pH 8.0). The column is washed with Buffer A and the bound protein is eluted with a gradient of 0 to 1 M NaCl. The properly refolded pneumolysin mutant should elute as a single peak between 13 and 20% Buffer B (25 mM Tris.HCl, 1 M NaCl, pH 8.0) similarly to what is observed for the wild-type. The protein peak is further analyzed by HPLC on a Superose 12 column and both elution time, aggregate/monomer ratio, and hemolytic activity are evaluated (see Table 4). The selected mutant(s) should present a single monomeric species with a Stokes radius comparable to the wild-type. Five clones (pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56) with high yields of monomeric modified polypeptides were selected for further analysis including nucleic acid sequencing. The amino and nucleic acid substitutions of these clones are shown in Tables 5A and 6. Throughout the specification and claims, proteins are given the name of the vector that encodes them.

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**Table 4. Comparison of Wild-Type (pNV19) And Mutant  
Pneumolysin Polypeptides**

Protein	Pure Monomer (mg/L)	HPLC (Elution time)	Hemolytic activity (U/mg)	Activity (% wild type)
pNV19	63	20.1	10 <sup>6</sup>	100
pNV111	92	19.3	2,555 (9) <sup>1</sup>	0.25
pNVJ22	86	20.7	2,440 (9)	0.24
pNVJ20	90	19.8	1,961 (6)	0.20
pNVJ1	66	20.2	1,536 (2)	0.15
pNVJ45	86	18.7	1,360 (5)	0.14
pNVJ56	104	19.8	2,000 (2)	0.20
pNV211	n.d.	20	1800 (2)	0.18
pNV207	100	20.5	800 (2)	0.08
pNV103	104.7	20	950 (2)	0.10

<sup>1</sup> Numbers in parenthesis indicate number of experiments.

**Table 5A: Amino Acid Sequence of Wild-Type (pNV19) Pneumolysin and Modified Forms**

[illegible]



Table 5B: Amino Acid Sequence of Modified Pneumolysin Polypeptides

Protein	Mutation	Hemolytic activity
pNV19	wild-type	100%
pNV21	446 P to S	25%
pNV46	286 E to D	12%
pNV22	243 G to R, 446 P to S	<1%
pNV38	243 G to V	<1%
pNV39	243 G to E	<1%
pNV40	243 G to S	<1%
pNV20	243 G to R	<1%



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**EXAMPLE 6****Site Directed Mutagenesis of Pneumolysin Gene With Single Mutation.**

To dissect whether a single mutation or multiple  
5 mutations are responsible for the loss of hemolytic  
activity in specific peptides (Table 4), each mutation was  
introduced into the wild-type allele as a single-site  
mutation using oligonucleotide directed mutagenesis.  
Table 7 presents the oligonucleotides used to introduce  
10 these specific mutations. Polypeptides carrying desired  
mutations were identified and their nucleic acid sequences  
confirmed. The following polypeptides with single base  
changes that resulted in a loss of hemolytic activity from  
these site-directed polypeptides were identified (See  
15 Table 5A): nucleic acid sequence 103 contains a single  
base change at 583 from wild-type T to modified G (195-  
Phe→Val); nucleic acid sequence 207 contains a single  
base change at 583 from wild-type T to modified A (195-  
Phe→Ile); nucleic acid sequence 111 contains a single  
20 base change at 443 from wild-type T to modified A (148-  
Met→Lys); nucleic acid sequence 211 contains a single  
base change at 181 from wild-type T to modified C (61-  
Ser→Pro).

The polypeptides shown in Table 5B exhibited  
25 poor refolding yields, explaining their reduced hemolytic  
activity. Single mutations introduced into pneumolysin  
polypeptide at positions 243, 286 and 446 or a combination  
of substitutions introduced at positions 243 and 446  
produced species found exclusively in the insoluble

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fraction as inclusion bodies. Attempted refolding of these mutants yielded mostly aggregated species.

**Table 7. Modified Pneumolysin Sequences**

MUTATION POSITION	AA #	Primer Sequence
443	148	Forward 5'ggtcaggtcaataatgtcccagctagaaAgcagtatg 3'
	Met-Lys	Reverse 5'gctgtgagccgtgattttttcatactgcTttctagctg 3'
583	195	Forward 5'gcagattcagattgttaatGttaagcagatttattata 3'
	Phe-Ile	Reverse 5'atctgcttaaCattaacaatctgaatctgcttttcgcc 3'
583	195	Forward 5'cagattgttaatAttaagcagatttattatacagtcagc3'
	Phe-Val	Reverse 5'aatctgcttaaTattaacaatctgaatctgcttttcgcc3'
181	61	Forward 5'acaagtgatattCctgtaacagctaccaacgacagtcgc3'
	Ser-Pro	Reverse 5'agctgttacagGaatatcacttgatattgtcgacaagct3'

5

#### EXAMPLE 7

##### Expression and Purification of Modified Polypeptides.

These single mutated genes were cloned into expression vectors (pET-24a) individually to overexpress the modified polypeptides in *E. coli*. The expression level is ~40%. Novel purification and refolding processes were developed to purify these recombinant modified

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pneumolysins.

Pneumolysin expressed in *E. coli* cells harboring the expression vector pNV19 was isolated from inclusion bodies by resuspending and lysing the cells in TEN buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA pH 8.0), with an air driven cell disrupter (Stansted Fluid Power Ltd.) under a pressure of 8,000 psi. The cell lysate was centrifuged at 13,000 rpm at 4°C for 20 minutes; both pellet and supernatant were saved for isolation of soluble and aggregated pneumolysin, respectively. The inclusion bodies were washed three times with TEN buffer and stored at -70°C. Purification and subsequent refolding were achieved by solubilizing the inclusion bodies in an 8 M urea solution (freshly prepared in TEN buffer), followed by PEG-assisted refolding. Polypeptide solutions in 8 M urea (200 µg/ml) were diluted 10-fold by drop-wise addition to a refolding solution, consisting of 20 µM of PEG 8,000 in 25 mM Tris-HCl, pH 8.0, under constant stirring at 4°C. The sample was clarified and loaded into a DEAE-Sepharose Fast Flow ion exchange column (Pharmacia) equilibrated in 25mM Tris-HCl, pH 8.0. A gradient of 0-1 M NaCl was applied and pneumolysin containing fractions were identified by detection of hemolytic activity, as described below, and by SDS-PAGE. The purified fractions were concentrated by using an Amicon concentrator and PM30 membrane. Aliquots of purified polypeptide were tested for hemolytic activity, and analyzed by SDS-PAGE and size exclusion chromatography, using a Superose 12 column.

Hemolytic activity assays were conducted in U-

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bottom micro-titer plates using TBS (Tris buffered saline, pH 7.4) as an incubation buffer. Following a pre-incubation period of 5 minutes with 1 mM DTT, twofold serial dilutions of normalized proteins were performed and the samples incubated with an identical volume of a 1% suspension of washed sheep erythrocytes (total volume 200  $\mu$ l) (Cappel) resuspended in the same buffer. The reactions were conducted at 37°C for 30 minutes and the extent of erythrocyte lysis was monitored spectrophotometrically by spinning down the U-plates transferring the supernatant to flat-bottomed plates and measuring the extent of hemoglobin release at 450 nm. The end point was set to be the concentration at which 50% lysis occurred and was based on comparison with a 0.5% cell suspension that was lysed hypotonically (see Tables 4 and 5B).

Another method of assaying the modified pneumolysin polypeptides is to conduct a hemolysis inhibition assay of the modified polypeptides. This assay consists of determining the ability of the mutant proteins to reduce or eliminate the hemolytic activity of the wild-type protein by pre-incubating erythrocytes with the modified pneumolysin polypeptides and assessing the hemolytic titer of the wild-type pneumolysin toward the pre-treated erythrocytes. The results from using this assay with four modified polypeptides are given in Table 8, and a detailed description of the procedure appears in Example 11.

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**Table 8. Hemolysis inhibition assay of pneumolysin by the pneumolysin mutants**

Designation	Mutation	End point (*)
pNV19	wild-type	512
pNV103	Phe <sup>195</sup> Val	64
pNV111	Met <sup>148</sup> Lys	128
pNV207	Phe <sup>195</sup> Ile	32
pNV211	Ser <sup>61</sup> Pro	512

(\*) Reciprocal of the hemolytic titer of a wild-type pneumolysin preparation in the presence of the indicated mutant

The antigenic cross-reactivity of the selected single site pneumolysin mutants was determined by immunizing rabbits (n=2) with each of the mutant proteins shown in Table 9 by conventional immunization procedures. Immunization of rabbits: New Zealand White rabbits (Covance, Denver, PA ) weighing 2-3 kg were immunized subcutaneously with 100 µg of wild-type or mutant pneumolysin emulsified with complete Freund's adjuvant, (Vol/Vol). Booster doses of vaccine mixed with incomplete Freund's adjuvant were administered by the same route and 42 days after the primary dose. Sera were collected on day 0, 21, 42, and 52. The sera were tested for the presence of antibodies against wild-type pneumolysin. The antigenic titer of pooled sera (n=2) towards type 14 pneumolysin was determined by ELISA. In brief, plates were coated with wild-type pneumolysin and incubated with

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serial dilutions of each of the anti-mutant pneumolysin sera. Significant binding of wild-type pneumolysin to antibodies elicited by the modified pneumolysin polypeptides was observed as shown in Table 9.

5

**Table 9. Reactivity and hemolysis neutralizing titer of mutant pneumolysin rabbit antisera towards type 14 pneumolysin by ELISA**

Designation	Mutation	Titer	Antibody Neutralizing Titer
pNV19	wild-type	892,647	256
pNV211	Ser <sup>61</sup> Pro	432,100	128
pNV111	Met <sup>148</sup> Lys	296,113	128
pNV103	Phe <sup>125</sup> Val	2,505,208	512
pNV207	Phe <sup>125</sup> Ile	402,426	128
PBS	-	-	8

As can be seen in Table 9, antisera to each of the above polypeptides, in addition to their strong cross-reaction with the wild-type pneumolysin as measured by  
 10 ELISA, have significant neutralizing, anti-hemolytic titers as measured in a hemolysis inhibition assay.



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**EXAMPLE 8****Preparation of Pneumolysoid Conjugates Preparation of polysaccharide for conjugation.**

5                   PnC type 14 polysaccharide (ATCC Lot #2016107) (390 mg) was dissolved in 16 ml of 0.5 N NaOH, and the solution was heated at 70°C for 3 hours. Following cooling of the solution, 1.93 ml of glacial acetic acid was added to bring the pH to 4. After addition of 3 ml of  
10 5% (w/v) NaNO<sub>2</sub>, the reaction mixture was kept stirring at 4°C for 2 hours. The sample was then diluted to 50 ml with deionized water and the pH was adjusted to 7 with 0.5 N NaOH. Excess reagents were dialyzed out by diafiltration with DI water through a Spectra/Por  
15 molecularporous membrane tubing (MWCOL:3,500), and the retentates freeze-dried. The deaminated type 14 polysaccharide was then molecular sieved on a Superdex G-200 (Pharmacia) column using PBS as eluent. Fractions eluting from the column with molecular weight between 5000  
20 and 15,000 as determined by Chromatography/Multiangle Laser Light Scattering using a Superose 12 column (Pharmacia) were pooled and dialyzed against DI water through a Spectra/Por molecularporous membrane tubing (MWCOL 3,500) and freeze-dried.

**25 Preparation of conjugates.**

Each of the PnCPS were first depolymerized and functional aldehydes were introduced into the fragmented CPS by oxidation with sodium metaperiodate. Following the oxidation process, the excess periodate was destroyed with

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ethylene glycol, the oxidized polysaccharides were dialysed against DI water and lyophilized.

Modified pneumolysin polypeptides in 0.2 M phosphate buffer (pH 8) at a concentration of 5 mg/ml were  
5 mixed with 2.5 equivalents (by weight) of PnC 14 polysaccharide-fragment together with 2 equivalents (by weight) of recrystallized sodium cyanoborohydride. Reaction mixtures were incubated at 37°C for 24 hours. Conjugates were then purified from the free components by  
10 passage through a Superdex G200 (Pharmacia) column using PBS containing 0.01% thimerosal as an eluent. Fractions eluting from the column were monitored on a Waters R403 differential refractometer and by UV spectroscopy at 280 nm. The fractions containing the conjugates were pooled,  
15 sterile-filtered through a 0.22 µm Millipore membrane and then stored at 4°C. Polypeptide and carbohydrate content were measured by the methods of Bradford and Dubois respectively. Polysaccharide content in the resulting conjugates were approximately 30%.

20 Tetanus toxoid conjugates for use as control, were also produced as described above and as follows: Tetanus toxoid (Serum Statens Institute) was first passed through a molecular sieve column (Superdex G-200 Pharmacia) in order to obtain the monomer form of the  
25 toxoid. For conjugation, 12 mg of the monomer and 36 mg of the PnC 14 polysaccharide-fragments were dissolved in 600 µl of 0.2 M phosphate buffer pH 7.2. Recrystallized sodium cyanoborohydride (24 mg) was then added to the solution which was then incubated at 37°C for 3-days. The

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conjugate was purified as above. The conjugates had polysaccharide contents in the 25-30% range (see Table 10).

**Table 10.**

**Composition of Tetanus-Toxoid and Modified Pneumolysin type 14 Conjugates**

Carrier Polypeptide	Approx. MW of PS	Polypeptide (mg/ml)	PS (mg/ml)	%PS in Conjugate
pNV103 #195 Phe-Val	9,000	0.170	0.079	32%
pNV207 #195 Phe-Ile	9,000	0.117	0.048	29%
pNV111 #148 Met-Lys	9,000	0.145	0.062	30%
pNV19 Wild-type	9,000	0.115	0.049	30%
Tetanus Tm	9,000	0.245	0.098	28%

5

**EXAMPLE 9****Immunization with Modified Pneumolysin Conjugates.**

Groups of 20 CD1 female mice (age 6-8 weeks), from Charles River Laboratories, were immunized subcutaneously (S.C.) with 2 µg of the conjugated polysaccharides of Example 8 adsorbed on aluminum (Aluminum hydroxide, Superfos, Denmark) at a concentration of 1 mg of elemental aluminum per ml of PBS containing 0.01% thimerosal. Mice received the vaccine at day 0, 28,

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and 49. Sera were collected at day 0, 42, and 59, and stored at -70°C.

#### **ELISA.**

Micro titer plates (Nunc Polysorb ELISA plates) were sensitized by adding 100 µl of type 14 polysaccharide-fragment (MW ca: 10,000)/HSA conjugate (2.5 µg/ml) in PBS. The plates were sealed and incubated at 37°C for 1 hour. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 0.5% (w/v) BSA in PBS for 1 hour at room temperature. The wells were then filled with 100 µl of serial two-fold dilutions in PBS-T plates, 100 µl of peroxidase labeled goat anti-mouse IgG (H+L) (Kirkegaard and Perry Laboratories), and then washed five times with PBS-T. Finally, 50 µl of TMB peroxidase substrate (Kirkegaard and Perry Laboratories) were added to each well, and following incubation of the plates for 10 minutes at room temperature, the reaction was stopped by the addition of 50 µl of 1 M H<sub>3</sub>PO<sub>4</sub>. The plates were read at 450 nm with a Molecular Device Amex microplate reader using 650 nm as a reference wavelength.

#### **Inhibition ELISA assay.**

Microtiter plates (NUNC Polysorp) were coated with PLY (20 ng in 100 µL to each well) in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) for one hour at 37°C. After washing the plates with PBS + 0.05% Tween 20 (PBST), the plates were post-coated with 150 µL of PBS + 0.1% BSA, rewashed, and stored at 4°C until used.

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Hyperimmune rabbit anti-PLY was diluted in PBST, added to the PLY coated plates, and incubated at room temperature for 1 h. After washing, 100  $\mu$ L of goat anti-rabbit Ig-HRP conjugate (KPL) diluted in PBSTween according to the manufacturer's instructions were added to each well. The plate was incubated at room temperature for one hour and then washed again. 100 $\mu$ L of TMB microwell substrate (KPL) were added to each well. The reaction was stopped after 10 minutes by the addition of TMB one-component stop solution (KPL) and the OD<sub>450 nm</sub> was immediately read. The dilution corresponding to 1/2 the maximum signal was chosen for the inhibition study. PLYD mutants as well as PLY as a control were diluted serially in three-fold ingrements in PBST containing the rabbit antiserum diluted such that the final mixture contained the dilution which gave half-maximal activity and applied immediately to the coated microtiter plates in duplicate. The plates were incubated at room temperature for one hour and processed. Inhibition was determined as percent of maximum signal achieved with dilute antiserum in the absence of any inhibitor.

**Opsonic activity of conjugate antisera.**

The opsonic ability of mice antisera to the PnC type 14 conjugates was tested in an *in vitro* opsonophagocytic killing assay using the human promyelocytic leukemia HL-60 cell line (ATCC #CCL240). (See Table 11). Briefly, 200 cfu of PnC type 14 (12-8-95 CB) cells were mixed in equal volume with serially diluted antibodies and incubated 15 minutes under shaking at 37°C

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in a 5% CO<sub>2</sub> incubator. Baby rabbit complement and HL-60 cells ( $5 \times 10^5$ ) cultured 5-days in the presence of 90 mM dimethylformamide were added to the mixture which was then incubated at 37°C for 1 hour under shaking. Aliquots were  
5 removed for quantitative culture and plated on chocolate agar. Titers were determined by extrapolating the antibody dilution corresponding to 50% live bacteria.

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**Table 11. Immunogenicity of PnC 14 Polysaccharide Conjugates**

Carrier Polypeptide	ELISA Titer at Day			Op+ Titer at Day	ELISA titer to wild-type pneumolysin at Day
	0	42	59		
Tetanus Toxoid	<50	287,000	170,000	28,000	<50
pNV103 #195 Phe-Val	<50	209,000	178,000	18,000	124,000
pNV207 #195 Phe-Ile	<50	175,000	149,000	31,000	111,000
pNV111 #148 Met-Lys	<50	137,000	127,000	10,500	84,000
pNV19 Wild-type	<50	275,000	241,000	29,000	124,000
PBS	<50	<50	<50	<100	

\* PnC 14 polysaccharide-specific antibody titer

+ Opsonophagocytic Titer

As can be seen from the data in Table 11, all of the modified pneumolysin conjugates elicited antibodies which had opsonophagocytic activity in the presence of complement. Mice immunized with all the above conjugates,

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in addition to a strong IgG anti-PS response, mount a very strong IgG response against the pneumolysoid carriers and to the same extent as that raised against the conjugated wild-type pneumolysin.

5

**Example 10**

**Preparation of tetravalent 6B/14/19F/23F pneumolysoid vaccines.**

**Preparation of conjugates.**

The hydrolysis of polysaccharides was carried  
10 out as follows: type 6B PS was depolymerized with 0.1 N HCl at 60°C for 3 hrs and 45 min; type 14 was depolymerized with 0.1 N HCl at 60°C for 7 hrs ; type 19F was depolymerized with a 10 mM NaOAc buffer of pH 4.1 at 70°C for 2 hrs and 20 min; and type 23F was depolymerized  
15 with 0.2M acetic acid solution at 100°C for 30 minutes.

Oxidized 6B PS was prepared as follows: the partially depolymerized PS ( 35 mg) was dissolved in 1750 ml DI water and treated with 250 ml of 10 mM NaIO<sub>4</sub> in the dark for 2 hrs at room temperature. The excess NaIO<sub>4</sub> was  
20 destroyed with ethylene glycol, and after extensive dialysis the oxidized PS was lyophilized. Oxidized 14 PS was prepared as described above for type 6B. Oxidized 19F was prepared as follows: 50 mg of depolymerized PS was dissolved in 0.2 M sodium phosphate buffer pH 7.5 (5ml)  
25 and treated with 41 ml of 100 mM NaIO<sub>4</sub> at 4°C overnight in the dark. Excess NaIO<sub>4</sub> destroyed with ethylene glycol and after extensive dialysis the oxidized 19F PS was lyophilized. Oxidized 23F was prepared as follows: 68 mg



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of partially depolymerized PS was dissolved in 3.4 ml of 3 mM NaIO<sub>4</sub> solution at room temperature in the dark for 1 hour. The excess NaIO<sub>4</sub> was destroyed by the addition of ethylene glycol, and after extensive dialysis, the  
5 oxidized PS was lyophilized to dryness.

The oxidized PSs were separately coupled to recombinant pneumolysoid mutant 207 in which amino acid Phe residue 195 was replaced by Ile. In brief, the oxidized PSs and the protein (5 mg/ml) in 0.2 M sodium  
10 phosphate buffer were combined at a PS/protein ratio of about 2.5:1 by weight at room temperature and sodium cyanoborohydride (2 equivalents by weight) was then added. The conjugation mixtures were incubated at 37°C for 2 days. After reduction of the residual aldehydes of the  
15 conjugated PS, with excess NaBH<sub>4</sub>, the conjugates were purified from the reaction mixtures by passage through a column of Superdex 200 PG (Pharmacia) eluted with PBS containing 0.01% thimerosal as the preservative, except for the type 23 conjugate where the conjugate was loaded  
20 onto a Q Sepharose Fast Flow column, and eluted with 10 mM Tris-HCl, pH 7.5 using a gradient of 0.5 M NaCl. Fractions corresponding to the conjugates were pooled and analyzed for protein and carbohydrate content as described in example 8 (see Table 12).

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**Table 12. Composition of pneumolysoid conjugates**

Pneumococcal Serotype	Approx. MW of PS	Polypeptide (mg/ml)	PS (mg/ml)	% PS in conjugate
6B	41,000	0.24	0.14	37
14	41,000	0.13	0.08	38
19F	10,000	0.46	0.14	23
23F	90,000	0.44	0.06	12

**Example 11****Immunization with pneumolysoid tetravalent vaccines.****Immunization of mice.**

5                Six to 8 weeks old female outbred CD-1 mice  
                  (Charles River, Raleigh) were immunized with monovalent  
                  or tetravalent vaccines. Streptococcus pneumoniae  
                  polysaccharides types 6B, 14, 19, and 23 were conjugated  
                  to tetanus toxoid or pneumolysin mutant (0.5 µg PS/0.2 ml  
 10               to 2 µg PS/0.2 ml) in 1 mg/ml 1 alum. The vaccines were  
                  administered subcutaneously, on days 0, 28, and 49, and  
                  blood samples were collected on days 0, 14, 28, 38 and 59.  
                  ELISA titers against polysaccharides and the carrier  
                  protein were determined using HSA-PS conjugates and wild-  
 15               type pneumolysin (Figs. 8, 9 and 10). The opsonic  
                  activity of the sera was determined in a phagocytic assay  
                  using HL-60 cell line as described in Example 9 (Fig.  
                  11).

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**Hemolysis assay.**

The pneumolysin activity was assessed according to Paton et al. (1993) Infect. Immun. 40:548, with some modifications. In brief, on standard U-bottomed  
5 microtiter plates, wild-type and mutant pneumolysin proteins were twofold serially diluted in TBS (15 mM Tris, 0.15 M NaCl, pH 7.5) plus 1 mM DTT as cofactor, in a final volume of 100 µl. One hundred microliters of 1% sheep erythrocyte suspension in TBS were added and the reaction  
10 conducted at 37°C for 30 minutes. After spinning down the unlysed cells, the extent of the erythrocyte lysis was monitored in the supernatant at 405 nm using a microtiter plate reader. The end point of the assay was taken as the well in which 50% of erythrocytes were lysed, based on a  
15 0.5% cell suspension lysed hypotonically.

**Hemolysis inhibition assay of murine antisera.**

Inhibition of the hemolytic activity was tested according to Paton et al. (1993) Infect. Immun. 40:548, with some variations. Before dilution, the mouse antisera  
20 were treated twice with chloroform, to eliminate cholesterol. A twofold serial dilution of 50 µl of the mice antisera were performed and 50 µl of toxin stock solution at 4HU (hemolytic units) were added. The hemolytic activity of the toxins were assessed immediately  
25 before the neutralization assay. After 15 min incubation at 37°C to allow serum antibody to bind to the pneumolysin, 100 µl sheep red blood (1% in TBS) (ICN, Costa Mesa, CA) was added in each well. The plates were incubated 30 min at 37°C and the unlysed cells were

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pelleted by centrifugation. The extent of the erythrocyte lysis released in the supernatant was monitored at 405 nm using a microtiter plate reader. The antibody titers were taken as the highest dilutions of sera which gave complete  
5 inhibition of the hemolysis (Fig. 12).

**Hemolysis inhibition assay by modified pneumolysin.**

Modified pneumolysin polypeptides can be tested for their ability to inhibit the hemolytic activity of wild-type pneumolysin when pre-incubated with  
10 erythrocytes. A suspension of erythrocytes (3 ml) was incubated with 1  $\mu$ l (1 mg/ml) of each of the modified pneumolysin polypeptides for 10 min and the suspension added to wells of a microtiter plate containing serial dilutions of wild-type pneumolysin. The plate was  
15 incubated at 37 °C for 30 min and the hemolytic titer compared with a control incubation performed with normal erythrocytes. The selected mutants exert variable degrees of inhibition of the wild type hemolytic activity upon pre-incubation with erythrocytes (Fig. 13), suggesting  
20 that these mutants are capable of competing with the wild type for the binding site, but are unable to insert into membranes to form lytic channels. The mutants pNV103 and pNV207 represent the most effective inhibitors, followed by pNV111. The mutant pNV211 apparently does not exhibit  
25 such inhibition properties. Additional corroboration of the structural integrity and identity of the PLYD mutants is that most of their antigenicity is retained when compared to native PLY as shown in Figure 14.

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### **Circular dichroism (CD) spectroscopy.**

The secondary and tertiary structures of the free wild type and mutant pneumolysin and the respective conjugate were evaluated by circular dichroism (CD) spectroscopy in the far UV (180 to 250 nm) and near UV (250 to 350 nm) regions, respectively. Concentrated stock solutions of protein were dialyzed exhaustively against a buffer system comprised of 10 mM NaPO<sub>4</sub> (pH 8.0). Spectra of samples containing 1.0 mg/ml protein were recorded at 0.1 nm wavelength intervals on a JASCO Model 710 circular dichroism spectropolarimeter (JASCO, Easton, MD) employing a scan speed of 5 nm/min and average response time of 1 s. A minimum of four consecutive scans were accumulated and the average spectra stored. The temperature of the samples was maintained at 25 °C through the use of water-jacketed 0.01 cm and 1.0 cm pathlength cells in the far and near UV, respectively.

### **Fluorescence spectroscopy.**

Fluorescence measurements were performed on an SLM AMINCO-Bowman 8100 Series 2 spectrofluorometer. Fluorescence spectra of samples containing 100 µg/ml protein in 10 mM NaPO<sub>4</sub> (pH 7.5) were recorded over the range of 300 to 500 nm employing an excitation wavelength of 290 nm and slit widths of 2 nm. Temperature stability was maintained through use of a water-jacketed 1.0 cm quartz cuvette thermostatted at 25°C.

A comparison of the fluorescence spectra of wild type pneumolysin and selected mutants has been performed under experimental conditions in which these proteins

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adopt a native folded conformation. As evidenced in Fig. 15, the fluorescence spectra of all the proteins are characterized by a maximum emission intensity at ~345 nm, with somewhat higher amplitudes observed for the mutant proteins when compared to the wild type. Overall, the results indicate that all the proteins are in a native conformation, which is characterized by a significant number of Trp residues exposed to solvent. These results have been observed previously for perfringolysin, and are consistent with the presence of a Trp-rich cell-binding domain in the C-terminus of these cytolysins.

**Basic structural and immunological features of pneumolysin (PLY), pneumolysoid (PLYD) and CPS-PLYD conjugates as assessed by circular dichroism.**

PLY overexpressed in *E. coli* and refolded from inclusion bodies exhibits a typical far UV CD spectrum characteristic of a high content of  $\beta$ -sheets with a minimum observed at ~ 215 nm (Minetti et al. *Biophys. J.*, 1998, 74, A233) which does not significantly change in the single point mutation PLYD. Likewise, chemical conjugation of either PLY or PLYD with PnCPS does not affect the overall secondary structure of the proteins (Figure 16A). The near UV CD spectrum (Figure 16B) which derives from the relative asymmetry of tyrosyl and tryptophanyl residues in the protein has also been assessed in the free versus conjugate protein and reveals a highly ordered structure resembling the wild type free protein. The conjugate, however exhibits minor changes in the near UV CD profile as a result from the presence of

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the polysaccharide on the surface of these complexes, interfering particularly with the specific Tyr signal (i.e., negative ellipticity with a minimum centered at ~ 280 nm), reduced in the respective conjugate. Additional  
5 corroboration of the structural integrity and identity of the PLYD mutants is that most of their antigenicity is retained when compared to native PLY as shown in Figure 14.

Spectroscopic methods represent a powerful tool  
10 in the evaluation of the integrity of proteins. In the particular case of conjugate vaccines which employ proteins as carriers, these methods, in conjunction with functional and immunological techniques may facilitate monitoring batch-to-batch variations as well as the  
15 molecular basis for vaccine efficacy (Crane et al. *Eur. J. Biochem.* 1997, 246, 320-327; Jones et al. *Dev. Biol. Stand.* 1996, 87, 143-151). The mutations render the protein atoxic, but it retains the ability to refold to a native-like structure, indistinguishable from the parent  
20 molecule. The nearly superimposable far UV CD spectra of the free mutant protein (i.e., pNV207) and the corresponding Pn 14 conjugate, as seen by both amplitude and crossover points, are indications that the secondary structure of the protein within this macromolecular  
25 complex remains intact. These results contrast with previous studies conducted with other polysaccharide-protein conjugates in which light variations in the secondary structure were noticeable, following conjugation (Crane et al. *Eur. J. Biochem.* 1997, 246, 320-327).

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The tyrosyl residues in the vicinity of the conjugation sites may be perturbed by the presence of the polysaccharide as indicated by the differences observed in the near UV CD spectra in the region around 280 nm.

5 However, the tryptophanyl peak, characteristic at 290 nm, remains unaffected by the conjugation, another indication that the Tyr-containing regions are not affected by the reductive amination procedure.

Overall the spectroscopic in conjunction with  
10 the serological results provide excellent evidence that PLYD-CPS conjugates represent suitable vaccine candidates for the prevention of pneumococcal diseases.

#### **Immunogenicity time course studies.**

An immunogenicity time course study for the  
15 tetravalent PLYD-PnCPS was carried out and is shown in Figure 17A. The animals received three injections at days 0, 28 and 49, and blood samples were obtained at days 0, 14, 28, 38, and 59. Each dose contained 0.5µg PnCPS of each type. The PnCPS-specific IgG response to each type  
20 increased over time to peak just after the second injection (titers ranging between 20,000 and 50,000) and then reached a plateau. Significant booster effects were observed after the second injection.

In Figure 17B is shown the time course of the  
25 PnCPS-specific IgG response of the tetravalent TT conjugate. Like for the PLYD combination vaccine, the animals were similarly immunized using the same schedule and same dose of vaccine. Again, the IgG response to each type polysaccharide increased after each injection with



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similar magnitude (final titers between 50,000 and 200,000) except for type 23F which gave a significantly lower titer (ca: 5,000). Booster effects were also observed for each type after the second injection, except  
5 for 23 which gave a much less pronounced effect.

For comparison with the above PLYD-PnCPS combination vaccine, the immunogenicity time course of the monovalent PLYD-PnCPS conjugates are shown in Figure 17C. The animals received the same doses of 0.5 $\mu$ g of PnCPS and  
10 had the same immunization schedule as mentioned above. The time course of the IgG response to the PnCPS in those monovalent conjugates was almost identical to the one observed for the combination except for the type 23 PnCPS which gave rise to a less steeper time course curve, with  
15 antibody titers an order of magnitude lower than those of the three other types.

The preclinical studies demonstrate that conjugates consisting of polysaccharides derived from four pneumococcal strains (6, 14, 19, and 23) and PLYD are  
20 highly immunogenic in animals, and they elicited PnCPS-specific antibodies titers which compared well with those raised with a TT tetravalent conjugate. In addition, the PLYD tetravalent conjugate was able to generate high levels of PLY-specific IgG antibodies that neutralized  
25 hemolytic activity of wild type PLY. In the light of a recently published report on the clear pathogenic role of PLY in the hearing loss and cochlear damage in a pneumococcal experimental meningitis model, it is clear that PLYD vaccine-induced antibodies will be a useful

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adjunct to the capsular antibodies to ameliorate or even prevent the feared complications associated with otitis media (Winter et al. Infection and Immunity 1997, 65, 4411-4418).

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We claim:

1. A modified pneumolysin polypeptide having attenuated hemolytic activity wherein said modified pneumolysin polypeptide is obtained by:
  - 5 a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid  
10 molecules in host cells;
  - b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
  - c) identifying the modified pneumolysin  
15 polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.
2. A modified pneumolysin polypeptide having attenuated hemolytic activity comprising an amino acid sequence of type 14 pneumolysin wherein at least one amino  
20 acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in attenuation of the hemolytic activity of the modified pneumolysin polypeptide.
- 25 3. The modified pneumolysin polypeptide according to claim 2, wherein the hemolytic activity is less than

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25% compared to wild-type pneumolysin.

4. A modified pneumolysin polypeptide according to claim 2 comprising at least one amino acid substitution in the amino acid sequence of Formula I at residue positions 61, 148, or 195 or the combination of substitutions at residue positions 33, 46, 83, 239 and 257

(Formula I)

```

10  Met Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala
    1             5             10
    Met Asn Tyr Asp Lys Lys Lys Leu Leu Thr His Gln
        15             20
    Gly Glu Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly
15  25             30             35
    Asn Gln Leu Pro Asp Glu Phe Val Val Ile Glu Arg
        40             45
    Lys Lys Arg Ser Leu Ser Thr Asn Thr Ser Asp Ile
        50             55             60
20  Ser Val Thr Ala Thr Asn Asp Ser Arg Leu Tyr Pro
        65             70
    Gly Ala Leu Leu Val Val Asp Glu Thr Leu Leu Glu
        75             80
    Asn Asn Pro Thr Leu Leu Ala Val Asp Arg Ala Pro
25  85             90             95
    Met Thr Tyr Ser Ile Asp Leu Pro Gly Leu Ala Ser
        100            105
    Ser Asp Ser Phe Leu Gln Val Glu Asp Pro Ser Asn
        110            115            120

```

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Ser Ser Val Arg Gly Ala Val Asn Asp Leu Leu Ala  
 125 130  
 Lys Trp His Gln Asp Tyr Gly Gln Val Asn Asn Val  
 135 140  
 5 Pro Ala Arg Met Gln Tyr Glu Lys Ile Thr Ala His  
 145 150 155  
 Ser Met Glu Gln Leu Lys Val Lys Phe Gly Ser Asp  
 160 165  
 Phe Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe  
 10 170 175 180  
 Asn Ser Val His Ser Gly Glu Lys Gln Ile Gln Ile  
 185 190  
 Val Asn Phe Lys Gln Ile Tyr Tyr Thr Val Ser Val  
 195 200  
 15 Asp Ala Val Lys Asn Pro Gly Asp Val Phe Gln Asp  
 205 210 215  
 Thr Val Thr Val Glu Asp Leu Lys Gln Arg Gly Ile  
 220 225  
 Ser Ala Glu Arg Pro Leu Val Tyr Ile Ser Ser Val  
 20 230 235 240  
 Ala Tyr Gly Arg Gln Val Tyr Leu Lys Leu Glu Thr  
 245 250  
 Thr Ser Lys Ser Asp Glu Val Glu Ala Ala Phe Glu  
 255 260  
 25 Ala Leu Ile Lys Gly Val Lys Val Ala Pro Gln Thr  
 265 270 275  
 Glu Trp Lys Gln Ile Leu Asp Asn Thr Glu Val Lys  
 280 285  
 Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly Ala

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	290					295						300
	Arg	Val	Val	Thr	Gly	Lys	Val	Asp	Met	Val	Glu	Asp
					305					310		
	Leu	Ile	Gln	Glu	Gly	Ser	Arg	Phe	Thr	Ala	Asp	His
5		315						320				
	Pro	Gly	Leu	Pro	Ile	Ser	Tyr	Thr	Thr	Ser	Phe	Leu
	325					330					335	
	Arg	Asp	Asn	Val	Val	Ala	Thr	Phe	Gln	Asn	Ser	Thr
				340					345			
10	Asp	Tyr	Val	Glu	Thr	Lys	Val	Thr	Ala	Tyr	Arg	Asn
	350						355					360
	Gly	Asp	Leu	Leu	Leu	Asp	His	Ser	Gly	Ala	Tyr	Val
					365					370		
	Ala	Gln	Tyr	Tyr	Ile	Thr	Trp	Asn	Glu	Leu	Ser	Tyr
15		375						380				
	Asp	His	Gln	Gly	Lys	Glu	Val	Leu	Thr	Pro	Lys	Ala
	385					390					395	
	Trp	Asp	Arg	Asn	Gly	Gln	Asp	Leu	Thr	Ala	His	Phe
				400						405		
20	Thr	Thr	Ser	Ile	Pro	Leu	Lys	Gly	Asn	Val	Arg	Asn
	410						415					420
	Leu	Ser	Val	Lys	Ile	Arg	Glu	Cys	Thr	Gly	Leu	Ala
					425					430		
	Trp	Glu	Trp	Trp	Arg	Thr	Val	Tyr	Glu	Lys	Thr	Asp
25		435						440				
	Leu	Pro	Leu	Val	Arg	Lys	Arg	Thr	Ile	Ser	Ile	Trp
	445					450					455	
	Gly	Thr	Thr	Leu	Tyr	Pro	Gln	Val	Glu	Asp	Lys	Val
				460						465		

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Glu Asn Asp

470

5. The modified pneumolysin according to claim 2 wherein the substituted amino acids are selected from the group consisting of proline or hydroxyproline for position 61; lysine arginine or histidine for position 148 and leucine, glycine, alanine, isoleucine or valine for position 195.
6. The modified pneumolysin according to claim 2 wherein the substituted amino acids are selected from the group consisting of serine, threonine, asparagine, glutamine, tyrosine or cystine for positions 33, 46 and 83; lysine arginine or histidine for position 239 and leucine, glycine, alanine, isoleucine or valine for position 255.
7. Modified pneumolysin polypeptide pNVJ1.
8. Modified pneumolysin polypeptide pNVJ20
9. Modified pneumolysin polypeptide pNVJ22.
10. Modified pneumolysin polypeptide pNVJ45.
11. Modified pneumolysin polypeptide pNVJ56.
12. Modified pneumolysin polypeptide pNV103.
13. Modified pneumolysin polypeptide pNV207
14. Modified pneumolysin polypeptide pNV111.

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15. Modified pneumolysin polypeptide pNV211.
16. A recombinant nucleic acid molecule comprising a sequence encoding the modified pneumolysin polypeptides of any one of claims 1 to 15.
- 5 17. A recombinant nucleic acid molecule comprising the following pneumolysin nucleic acid sequence:

	ATGGCAAATA AAGCAGTAAA TGACTTTATA CTAGCTATGA	40
	ATTACGATAA AAAGAACTC TTGACCCATC AGGGAGAAAG	80
	TATTGAAAAT CGTTTCATCA AAGAGGGTAA TCAGCTACCC	120
10	GATGAGTTTG TTGTTATCGA AAGAAAGAAG CGGAGCTTGT	160
	CGACAAATAC AAGTGATATT TCTGTAACAG CTACCAACGA	200
	CAGTCGCCTC TATCCTGGAG CACTTCTCGT AGTGGATGAG	240
	ACCTTGTTAG AGAATAATCC CACTCTTCTT GCGGTCGATC	280
	GTGCTCCGAT GACTTATAGT ATTGATTTGC CTGGTTTGGC	320
15	AAGTAGCGAT AGCTTTCTCC AAGTGGAAGA TCCCAGCAAT	360
	TCAAGTG TTC GCGGAGCGGT AAACGATTTG TTGGCTAAGT	400
	GGCATCAAGA TTATGGTCAG GTCAATAATG TCCCAGCTAG	440
	AATGCAGTAT GAAAAAATCA CGGCTCACAG CATGGAACAA	480
	CTCAAGGTCA AGTTTGGTTC TGACTTTGAA AAGACAGGGA	520
20	ATTCTCTTGA TATTGATTTT AACTCTGTCC ATTCAGGCGA	560
	AAAGCAGATT CAGATTGTTA ATTTTAAGCA GATTTATTAT	600
	ACAGTCAGCG TAGACGCTGT TAAAAATCCA GGAGATGTGT	640
	TTCAAGATAC TGTAACGGTA GAGGATTTAA AACAGAGAGG	680
	AATTTCTGCA GAGCGTCCTT TGGTCTATAT TTCGAGTGTT	720
25	GCTTATGGGC GCCAAGTCTA TCTCAAGTTG GAAACCACGA	760
	GTAAGAGTGA TGAAGTAGAG GCTGCTTTTG AAGCTTTGAT	800
	AAAAGGAGTC AAGGTAGCTC CTCAGACAGA GTGGAAGCAG	840



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	ATTTTGGACA ATACAGAAGT GAAGGCGGTT ATTTTAGGGG	880
	GCGACCCAAG TTCGGGTGCC CGAGTTGTAA CAGGCAAGGT	920
	GGATATGGTA GAGGACTTGA TTCAAGAAGG CAGTCGCTTT	960
	ACAGCAGATC ATCCAGGCTT GCCGATTTCC TATACAACTT	1000
5	CTTTTTTACG TGACAATGTA GTTGCGACCT TTCAAAATAG	1040
	TACAGACTAT GTTGAGACTA AGGTTACAGC TTACAGAAAC	1080
	GGAGATTTAC TGCTGGATCA TAGTGGTGCC TATGTTGCCC	1120
	AATATTATAT TACTTGGAAT GAATTATCCT ATGATCATCA	1160
	AGGTAAGGAA GTCTTGACTC CTAAGGCTTG GGACAGAAAT	1200
10	GGGCAGGATT TAACGGCTCA CTTTACCACT AGTATTCCTT	1240
	TAAAAGGGAA TGTTCGTAAT CTCTCTGTCA AAATTAGAGA	1280
	GTGTACCGGG CTTGCTTGGG AATGGTGGCG TACGGTTTAT	1320
	GAAAAAACCG ATTTGCCACT AGTGCGTAAG CGGACGATTT	1360
	CTATTTGGGG AACAACTCTC TATCCGCAGG TAGAAGATAA	1400
15	GGTAGAAAAT GAC	1413

and wherein said nucleic acid sequence comprises one or more of the nucleotide substitutions selected from the group consisting of:

A-50→G, G-54→T, T-181→C, A-196→T and T-302→C;

20 or

A-122→G, A-514→G, T-583→A and A-764→G;

or

A-187→T, T-380→A, A-382→C and T-443→A;

or

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T-98→C, T-137→C, T-248→C, T-717→A and A-770→G;

or

T-134→C, A-305→G, A-566→G and T-583→G.

18. A recombinant nucleic acid molecule comprising the  
5 sequence shown in Figure 1, further comprising the  
following nucleic acid substitution: T-583→G
19. A recombinant nucleic acid molecule comprising the  
sequence shown in Figure 1, further comprising the  
following nucleic acid substitution: T-583→A.
- 10 20. A recombinant nucleic acid molecule comprising the  
sequence shown in Figure 1, further comprising the  
following nucleic acid substitution: T-443→A.
21. A recombinant nucleic acid molecule comprising the  
sequence shown in Figure 1, further comprising the  
15 following nucleic acid substitution: T-181→C.
22. The recombinant nucleic acid molecules of any one of  
claims 16 to 21 as contained in a vector such as a  
plasmid, cosmid, bacteriophage or yeast artificial  
chromosome.
- 20 23. A microorganism comprising the nucleic acid molecule  
of any one of claims 16 to 22.
24. The microorganism according to claim 23 wherein the  
microorganism is selected from the group consisting

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of bacteria, yeast, mammalian or insect cells.

25. The microorganism according to claim 23 wherein the microorganism is *E. coli*.
26. The modified pneumolysin polypeptides of any one of claims 1 to 15 wherein the polypeptides are conjugated with bacterial polysaccharide.
27. The modified pneumolysin conjugates of claim 26 wherein the bacterial polysaccharide is from a bacteria selected from the group consisting of a *Haemophilus influenzae* type b; meningococcal group A, B or C; group B streptococcus types Ia, Ib, II, III, V or VIII and pneumococcal.
28. A vaccine comprising at least one of the polypeptides of claims 1 to 15 and a pharmaceutically acceptable carrier.
29. The vaccine according to claim 28 wherein the polypeptide is conjugated to a bacterial polysaccharide.
30. The vaccine according to claim 29 wherein the bacterial polysaccharide is selected from the group consisting of *Haemophilus influenzae* type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus serotypes Ia, Ib, II, III, V, or VIII; or one or more of serotypes 1-23 of *S. pneumoniae*.

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31. A method for killing bacteria comprising contacting said bacteria with antibodies to an immunogenic molecule comprising the modified pneumolysin according to any one of claims 1 to 15 in the presence of complement.
32. The method according to claim 31 wherein the immunogenic molecule is a polysaccharide-polypeptide conjugate wherein the polysaccharide is a bacterial capsular polysaccharide.
33. A method for immunization of mammals comprising administering the vaccines of any one of claims 28 to 30 to said mammals.
34. A method for obtaining modified pneumolysin polypeptides having reduced hemolytic activity and being suitable for eliciting an immunogenetic response which is cross-reactive with wild-type pneumolysin comprising the steps of:
- a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid molecules in host cells;
  - b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
  - c) identifying the modified pneumolysin

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polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.

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ATGGCAAATA AAGCAGTAAA TGACTTTATA CTAGCTATGA	40
ATTACGATAA AAAGAAACTC TTGACCCATC AGGGAGAAAG	80
TATTGAAAAT CGTTTCATCA AAGAGGGTAA TCAGCTACCC	120
GATGAGTTTG TTGTTATCGA AAGAAAGAAG CGGAGCTTGT	160
CGACAAATAC AAGTGATATT TCTGTAACAG CTACCAACGA	200
CAGTCGCCTC TATCCTGGAG CACTTCTCGT AGTGGATGAG	240
ACCTTGTTAG AGAATAATCC CACTCTTCTT GCGGTCGATC	280
GTGCTCCGAT GACTTATAGT ATTGATTTGC CTGGTTTGGC	320
AAGTAGCGAT AGCTTTCTCC AAGTGGAAGA TCCCAGCAAT	360
TCAAGTGTTT GCGGAGCGGT AAACGATTTG TTGGCTAAGT	400
GGCATCAAGA TTATGGTCAG GTCAATAATG TCCCAGCTAG	440
AATGCAGTAT GAAAAAATCA CGGCTCACAG CATGGAACAA	480
CTCAAGGTCA AGTTTGGTTC TGACTTTGAA AAGACAGGGA	520
ATTCTCTTGA TATTGATTTT AACTCTGTCC ATTCAGGCGA	560
AAAGCAGATT CAGATTGTTA ATTTTAAGCA GATTTATTAT	600
ACAGTCAGCG TAGACGCTGT TAAAAATCCA GGAGATGTGT	640
TTCAAGATAC TGTAACGGTA GAGGATTTAA AACAGAGAGG	680
AATTTCTGCA GAGCGTCCTT TGGTCTATAT TTCGAGTGTT	720
GCTTATGGGC GCCAAGTCTA TCTCAAGTTG GAAACCACGA	760
GTAAGAGTGA TGAAGTAGAG GCTGCTTTTG AAGCTTTGAT	800
AAAAGGAGTC AAGGTAGCTC CTCAGACAGA GTGGAAGCAG	840
ATTTTGGACA ATACAGAAGT GAAGGCGGTT ATTTTAGGGG	880
GCGACCCAAG TTCGGGTGCC CGAGTTGTAA CAGGCAAGGT	920
GGATATGGTA GAGGACTTGA TTCAAGAAGG CAGTCGCTTT	960

**FIG. 1A**

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ACAGCAGATC ATCCAGGCTT GCCGATTTCC TATACAACTT	1000
CTTTTTTTACG TGACAATGTA GTTGCGACCT TTCAAAATAG	1040
TACAGACTAT GTTGAGACTA AGGTTACAGC TTACAGAAAC	1080
GGAGATTTAC TGCTGGATCA TAGTGGTGCC TATGTTGCCC	1120
AATATTATAT TACTTGGAAT GAATTATCCT ATGATCATCA	1160
AGGTAAGGAA GTCTTGACTC CTAAGGCTTG GGACAGAAAT	1200
GGGCAGGATT TAACGGCTCA CTTTACCACT AGTATTCCTT	1240
TAAAAGGGAA TGTTCGTAAT CTCTCTGTCA AAATTAGAGA	1280
GTGTACCGGG CTTGCTTGGG AATGGTGGCG TACGGTTTAT	1320
GAAAAAACCG ATTTGCCACT AGTGCGTAAG CGGACGATTT	1360
CTATTTGGGG AACAACTCTC TATCCGCAGG TAGAAGATAA	1400
GGTAGAAAAT GAC	1413

**FIG. 1B**

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ATGGCAAATA AAGCAGTAAA TGACTTTATA CTAGCTATGA	40
ATTACGATAN <sub>50</sub> AAAN <sub>54</sub> AAACTC TTGACCCATC AGGGAGAAAG	80
TATTGAAAAT CGTTTCAN <sub>98</sub> CA AAGAGGGTAA TCAGCTACCC	120
GN <sub>122</sub> TGAGTTTG TTGN <sub>134</sub> TAN <sub>137</sub> CGA AAGAAAGAAG CGGAGCTTGT	160
CGACAAATAC AAGTGATATT N <sub>181</sub> CTGTAN <sub>187</sub> CAG CTACCN <sub>196</sub> ACGA	200
CAGTCGCCTC TATCCTGGAG CACTTCTCGT AGTGGATGAG	240
ACCTTGTN <sub>248</sub> AG AGAATAATCC CACTCTTCTT GCGGTN <sub>276</sub> GATC	280
GTGCTCCGAT GACTTATAGT AN <sub>302</sub> TGN <sub>305</sub> TTTGC CTGGTTTGGC	320
AAGTAGCGAT AGCTTTCTCC AAGTGGAAGA N <sub>351</sub> CCCAGCAAT	360
TCAAGTGTTT GCGGAGCGGN <sub>380</sub> AN <sub>382</sub> ACGATTG TTGGCTAAGT	400
GGCATCAAGA TTATGGTCAG GTCAATAATG TCCCAGCTAG	440
AAAN <sub>443</sub> GCAGTAT GAAAAAATN <sub>459</sub> A CGGCTCACAG CATGGAACAA	480
CTCAAGGTCA AGTTTGGTTC TGAAGTTGAA AAGN <sub>514</sub> CAGGGA	520
ATTCTCTTGA TATTGATTTT AACTCTGTCC ATTCAGGN <sub>558</sub> GA	560
AAAGCN <sub>566</sub> GATT CAGATTGTTA ATN <sub>583</sub> TTAAGCA GATTTATTAT	600
ACAGTCAGCG TAGACGCTGT TAAAAATCCA GGAGATGTGT	640
TTCAAGATAC TGTAACGGTA GAGGATTTAA AACAGAGAGG	680
AATTTCTGCA GAGCGTCCTT TGGTCTATAT TTCGAGN <sub>717</sub> GTT	720
GCTTATGGGC GCCAAGTCTA TCTCAAGTTG GAAACCACGA	760
GTAN <sub>764</sub> GAGTGN <sub>770</sub> TGAAGTAGAG GCTGCTTTTG AAGCTTTGAT	800
AAAAGGAGTC AAGGTAGCTC CTCAGACAGA GTGGAAGCAG	840
ATTTTGGACA ATACAGAAGT GAAGGCGGTT ATTTTAGGGG	880
GCGACCCAAG TTCGGGTGCC CGAGTTGTAA CAGGCAAGGT	920
GGATATGGTA GAGGACTTGA TTCAAGAAGG CAGTCGCTTT	960
ACAGCAGATC ATCCAGGCTT GCCGATTTC TATACAACTT	1000

FIG. 2A



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CTTTTTTACG TGACAATGTA GTTGCGACCT TTCAAAAN <sub>1038</sub> AG	1040
TACAGACTAT GTTGAGACTA AGGTTACAGC TTACAGAAAC	1080
GGAGATTTAC TGCTGGATCA TAGTGGTGCC TATGTTGCCC	1120
AATATTATAT TACTTGGN <sub>1138</sub> AT GAATTATCCT ATGATCATCA	1160
AGGTAAGGAA GTCTTGACTC CTAAGGCTTG GGACAGAAAT	1200
GGGCAGGATT TN <sub>1212</sub> ACGGCTCA CTTTACCACT AGTATTCCTT	1240
TAAAAGGGAA TGTTCGTAAT CTCTCTGTCA AAATTAGAGA	1280
GTGTACCGGG CTTGCN <sub>1296</sub> TGGG AATGGTGGCG TACGGTTTAT	1320
GAAAAAACCG ATTTGCCACT AGTGCGTAAG CGGACGATTT	1360
CTATTTGGGG AACAACTCTC TATCCN <sub>1386</sub> CAGG TAGAN <sub>1395</sub> GATAA	1400
GGTAGAAAAT GAC	1413

FIG. 2B

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Met	Ala	Asn	Lys	Ala	Val	Asn	Asp	Phe	Ile	Leu	Ala	
1				5					10			
Met	Asn	Tyr	Asp	Lys	Lys	Lys	Leu	Leu	Thr	His	Gln	
		15					20					
Gly	Glu	Ser	Ile	Glu	Asn	Arg	Phe	Ile	Lys	Glu	Gly	
25				30						35		
Asn	Gln	Leu	Pro	Asp	Glu	Phe	Val	Val	Ile	Glu	Arg	
			40					45				
Lys	Lys	Arg	Ser	Leu	Ser	Thr	Asn	Thr	Ser	Asp	Ile	
	50					55				60		
Ser	Val	Thr	Ala	Thr	Asn	Asp	Ser	Arg	Leu	Tyr	Pro	
			65						70			
Gly	Ala	Leu	Leu	Val	Val	Asp	Glu	Thr	Leu	Leu	Glu	
		75				80						
Asn	Asn	Pro	Thr	Leu	Leu	Ala	Val	Asp	Arg	Ala	Pro	
85				90						95		
Met	Thr	Tyr	Ser	Ile	Asp	Leu	Pro	Gly	Leu	Ala	Ser	
			100					105				
Ser	Asp	Ser	Phe	Leu	Gln	Val	Glu	Asp	Pro	Ser	Asn	
	110				115					120		
Ser	Ser	Val	Arg	Gly	Ala	Val	Asn	Asp	Leu	Leu	Ala	
			125						130			
Lys	Trp	His	Gln	Asp	Tyr	Gly	Gln	Val	Asn	Asn	Val	
	135					140						
Pro	Ala	Arg	Met	Gln	Tyr	Glu	Lys	Ile	Thr	Ala	His	
145				150						155		
Ser	Met	Glu	Gln	Leu	Lys	Val	Lys	Phe	Gly	Ser	Asp	
			160					165				
Phe	Glu	Lys	Thr	Gly	Asn	Ser	Leu	Asp	Ile	Asp	Phe	
	170				175					180		
Asn	Ser	Val	His	Ser	Gly	Glu	Lys	Gln	Ile	Gln	Ile	
			185						190			
Val	Asn	Phe	Lys	Gln	Ile	Tyr	Tyr	Thr	Val	Ser	Val	
		195				200						
Asp	Ala	Val	Lys	Asn	Pro	Gly	Asp	Val	Phe	Gln	Asp	
205				210						215		
Thr	Val	Thr	Val	Glu	Asp	Leu	Lys	Gln	Arg	Gly	Ile	
			220					225				
Ser	Ala	Glu	Arg	Pro	Leu	Val	Tyr	Ile	Ser	Ser	Val	
	230				235					240		
Ala	Tyr	Gly	Arg	Gln	Val	Tyr	Leu	Lys	Leu	Glu	Thr	
			245					250				
Thr	Ser	Lys	Ser	Asp	Glu	Val	Glu	Ala	Ala	Phe	Glu	
		255				260						
Ala	Leu	Ile	Lys	Gly	Val	Lys	Val	Ala	Pro	Gln	Thr	
265				270						275		
Glu	Trp	Lys	Gln	Ile	Leu	Asp	Asn	Thr	Glu	Val	Lys	
			280					285				

FIG. 3A

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Ala	Val	Ile	Leu	Gly	Gly	Asp	Pro	Ser	Ser	Gly	Ala
290						295					300
Arg	Val	Val	Thr	Gly	Lys	Val	Asp	Met	Val	Glu	Asp
				305					310		
Leu	Ile	Gln	Glu	Gly	Ser	Arg	Phe	Thr	Ala	Asp	His
		315					320				
Pro	Gly	Leu	Pro	Ile	Ser	Tyr	Thr	Thr	Ser	Phe	Leu
325					330					335	
Arg	Asp	Asn	Val	Val	Ala	Thr	Phe	Gln	Asn	Ser	Thr
			340					345			
Asp	Tyr	Val	Glu	Thr	Lys	Val	Thr	Ala	Tyr	Arg	Asn
350					355						360
Gly	Asp	Leu	Leu	Leu	Asp	His	Ser	Gly	Ala	Tyr	Val
				365					370		
Ala	Gln	Tyr	Tyr	Ile	Thr	Trp	Asn	Glu	Leu	Ser	Tyr
		375					380				
Asp	His	Gln	Gly	Lys	Glu	Val	Leu	Thr	Pro	Lys	Ala
385					390					395	
Trp	Asp	Arg	Asn	Gly	Gln	Asp	Leu	Thr	Ala	His	Phe
			400					405			
Thr	Thr	Ser	Ile	Pro	Leu	Lys	Gly	Asn	Val	Arg	Asn
410						415					420
Leu	Ser	Val	Lys	Ile	Arg	Glu	Cys	Thr	Gly	Leu	Ala
				425					430		
Trp	Glu	Trp	Trp	Arg	Thr	Val	Tyr	Glu	Lys	Thr	Asp
		435					440				
Leu	Pro	Leu	Val	Arg	Lys	Arg	Thr	Ile	Ser	Ile	Trp
445					450					455	
Gly	Thr	Thr	Leu	Tyr	Pro	Gln	Val	Glu	Asp	Lys	Val
			460					465			
Glu	Asn	Asp									
470											

FIG. 3B

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Met	Ala	Asn	Lys	Ala	Val	Asn	Asp	Phe	Ile	Leu	Ala	
1				5					10			
Met	Asn	Tyr	Asp	Xaa	Xaa	Lys	Leu	Leu	Thr	His	Gln	
		15					20					
Gly	Glu	Ser	Ile	Glu	Asn	Arg	Phe	Xaa	Lys	Glu	Gly	
25					30					35		
Asn	Gln	Leu	Pro	Xaa	Glu	Phe	Val	Xaa	Xaa	Glu	Arg	
			40					45				
Lys	Lys	Arg	Ser	Leu	Ser	Thr	Asn	Thr	Ser	Asp	Ile	
	50					55					60	
Xaa	Val	Xaa	Ala	Thr	Xaa	Asp	Ser	Arg	Leu	Tyr	Pro	
			65						70			
Gly	Ala	Leu	Leu	Val	Val	Asp	Glu	Thr	Xaa	Leu	Glu	
		75					80					
Asn	Asn	Pro	Thr	Leu	Leu	Ala	Val	Asp	Arg	Ala	Pro	
85					90					95		
Met	Thr	Tyr	Ser	Xaa	Xaa	Leu	Pro	Gly	Leu	Ala	Ser	
			100					105				
Ser	Asp	Ser	Phe	Leu	Gln	Val	Glu	Asp	Pro	Ser	Asn	
	110					115					120	
Ser	Ser	Val	Arg	Gly	Ala	Xaa	Xaa	Asp	Leu	Leu	Ala	
			125						130			
Lys	Trp	His	Gln	Asp	Tyr	Gly	Gln	Val	Asn	Asn	Val	
		135					140					
Pro	Ala	Arg	Xaa	Gln	Tyr	Glu	Lys	Xaa	Thr	Ala	His	
145					150					155		
Ser	Met	Glu	Gln	Leu	Lys	Val	Lys	Phe	Gly	Ser	Asp	
			160					165				
Phe	Glu	Lys	Xaa	Gly	Asn	Ser	Leu	Asp	Ile	Asp	Phe	
	170				175						180	
Asn	Ser	Val	His	Ser	Gly	Glu	Lys	Xaa	Ile	Gln	Ile	
			185						190			
Val	Asn	Xaa	Lys	Gln	Ile	Tyr	Tyr	Thr	Val	Ser	Val	
		195					200					
Asp	Ala	Val	Lys	Asn	Pro	Gly	Asp	Val	Phe	Gln	Asp	
205					210					215		
Thr	Val	Thr	Val	Glu	Asp	Leu	Lys	Gln	Arg	Gly	Ile	
			220					225				
Ser	Ala	Glu	Arg	Pro	Leu	Val	Tyr	Ile	Ser	Xaa	Val	
	230					235					240	
Ala	Tyr	Xaa	Arg	Gln	Val	Tyr	Leu	Lys	Leu	Glu	Thr	
			245						250			
Thr	Ser	Xaa	Ser	Xaa	Glu	Val	Glu	Ala	Ala	Phe	Glu	
		255					260					
Ala	Leu	Ile	Lys	Gly	Val	Lys	Val	Ala	Pro	Gln	Thr	
265					270					275		
Glu	Trp	Lys	Gln	Ile	Leu	Asp	Asn	Thr	Xaa	Val	Lys	
			280					285				

FIG. 4A

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Ala	Val	Ile	Leu	Gly	Gly	Asp	Pro	Ser	Ser	Gly	Ala
290						295					300
Arg	Val	Val	Thr	Gly	Lys	Val	Asp	Met	Val	Glu	Asp
				305						310	
Leu	Ile	Gln	Glu	Gly	Ser	Arg	Phe	Thr	Ala	Asp	His
		315					320				
Pro	Gly	Leu	Pro	Ile	Ser	Tyr	Thr	Thr	Ser	Phe	Leu
325					330					335	
Arg	Asp	Asn	Val	Val	Ala	Thr	Phe	Gln	Asn	Ser	Thr
			340					345			
Asp	Tyr	Val	Glu	Thr	Lys	Val	Thr	Ala	Tyr	Arg	Asn
350						355					360
Gly	Asp	Leu	Leu	Leu	Asp	His	Ser	Gly	Ala	Tyr	Val
				365					370		
Ala	Gln	Tyr	Tyr	Ile	Thr	Trp	Xaa	Glu	Leu	Ser	Tyr
		375					380				
Asp	His	Gln	Gly	Lys	Glu	Val	Leu	Thr	Pro	Lys	Ala
385					390					395	
Trp	Asp	Arg	Asn	Gly	Gln	Asp	Leu	Thr	Ala	His	Phe
			400					405			
Thr	Thr	Ser	Ile	Pro	Leu	Lys	Gly	Asn	Val	Arg	Asn
	410					415					420
Leu	Ser	Val	Lys	Ile	Arg	Glu	Cys	Thr	Gly	Leu	Ala
				425					430		
Trp	Glu	Trp	Trp	Arg	Thr	Val	Tyr	Glu	Lys	Thr	Asp
		435					440				
Leu	Xaa	Leu	Val	Arg	Lys	Arg	Thr	Ile	Ser	Ile	Trp
445					450					455	
Gly	Thr	Thr	Leu	Tyr	Pro	Gln	Val	Glu	Asp	Lys	Val
			460					465			
Glu	Asn	Asp									
	470										

FIG. 4B

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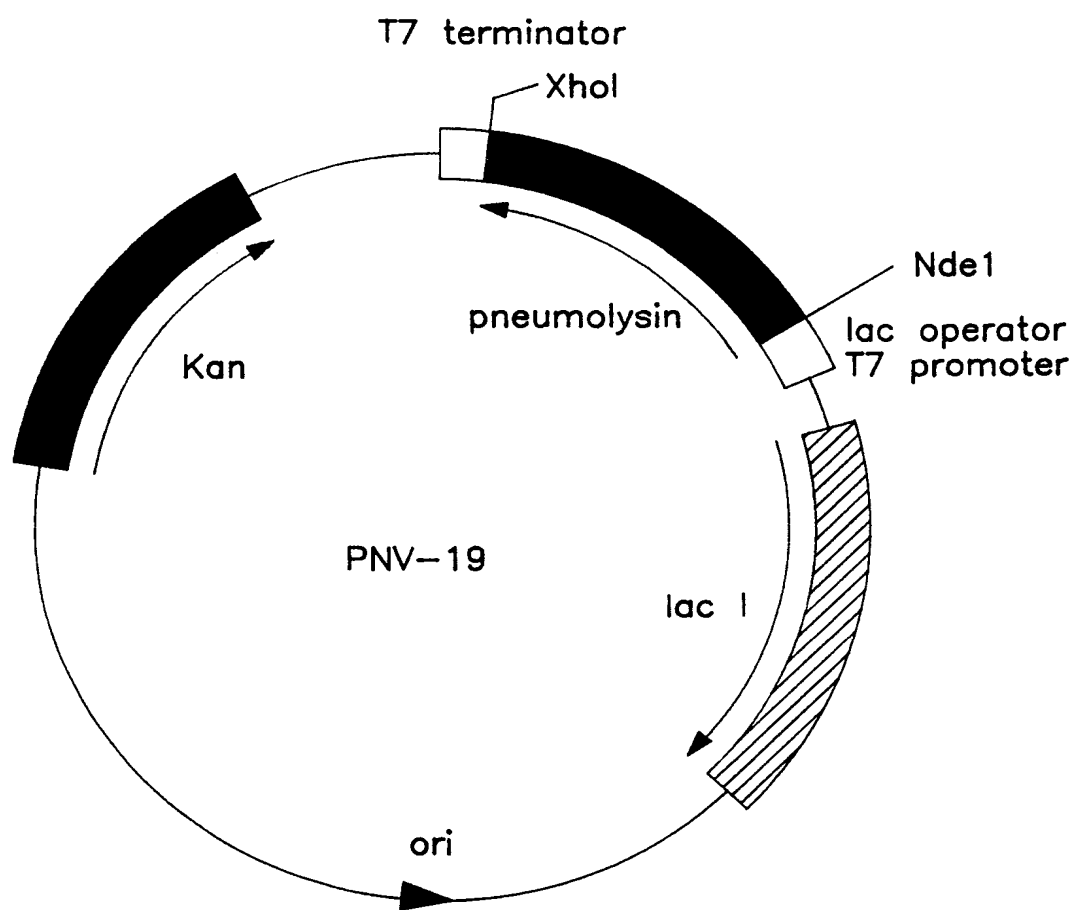


FIG. 5

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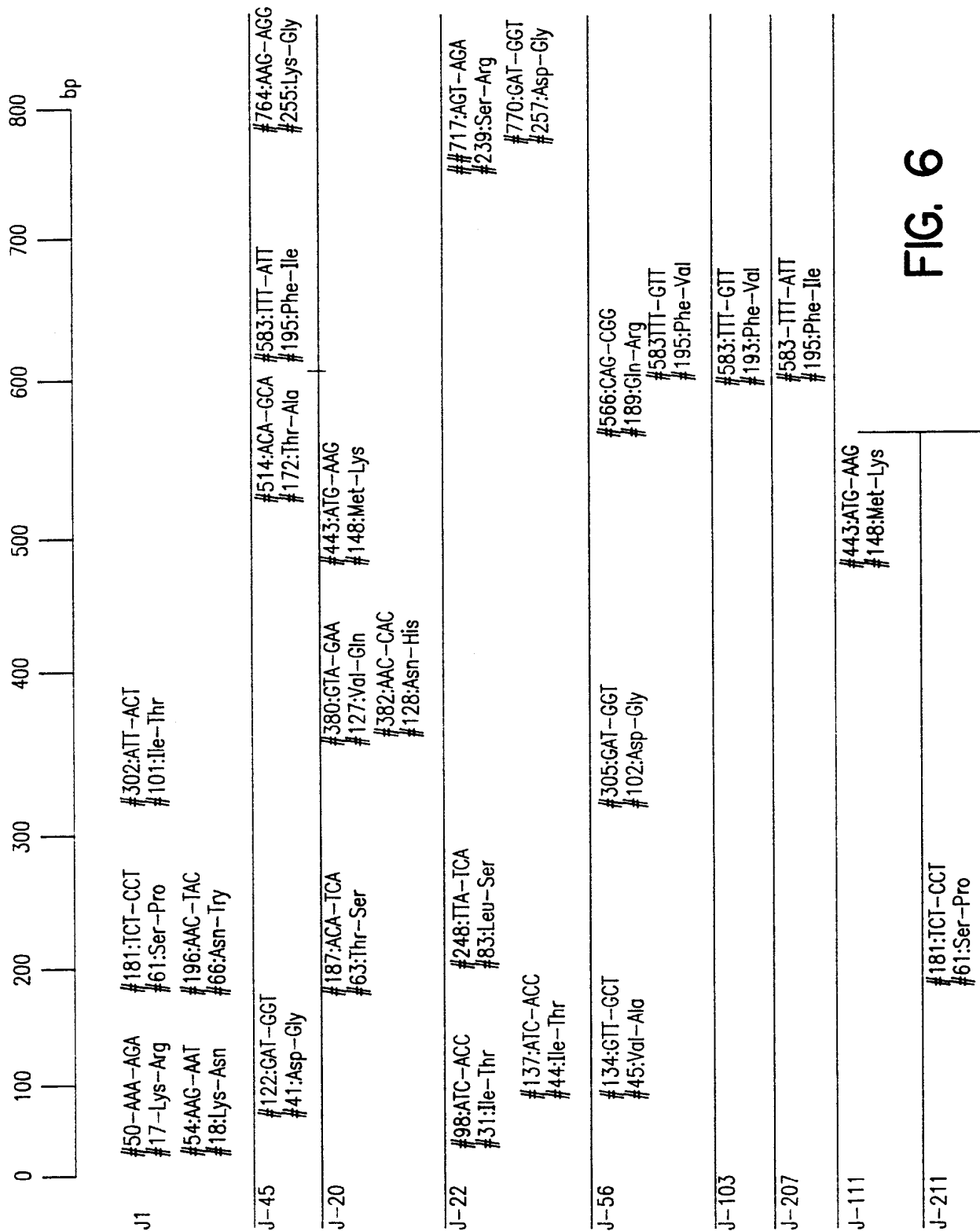
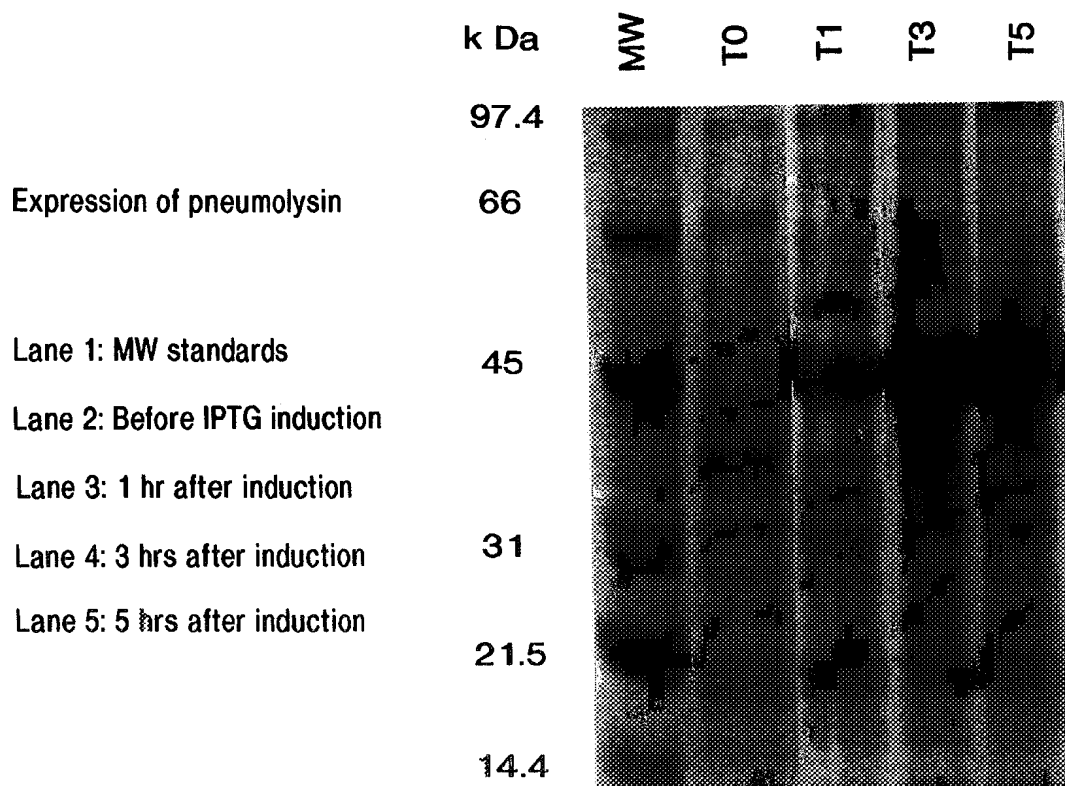


FIG. 6

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**FIG. 7**



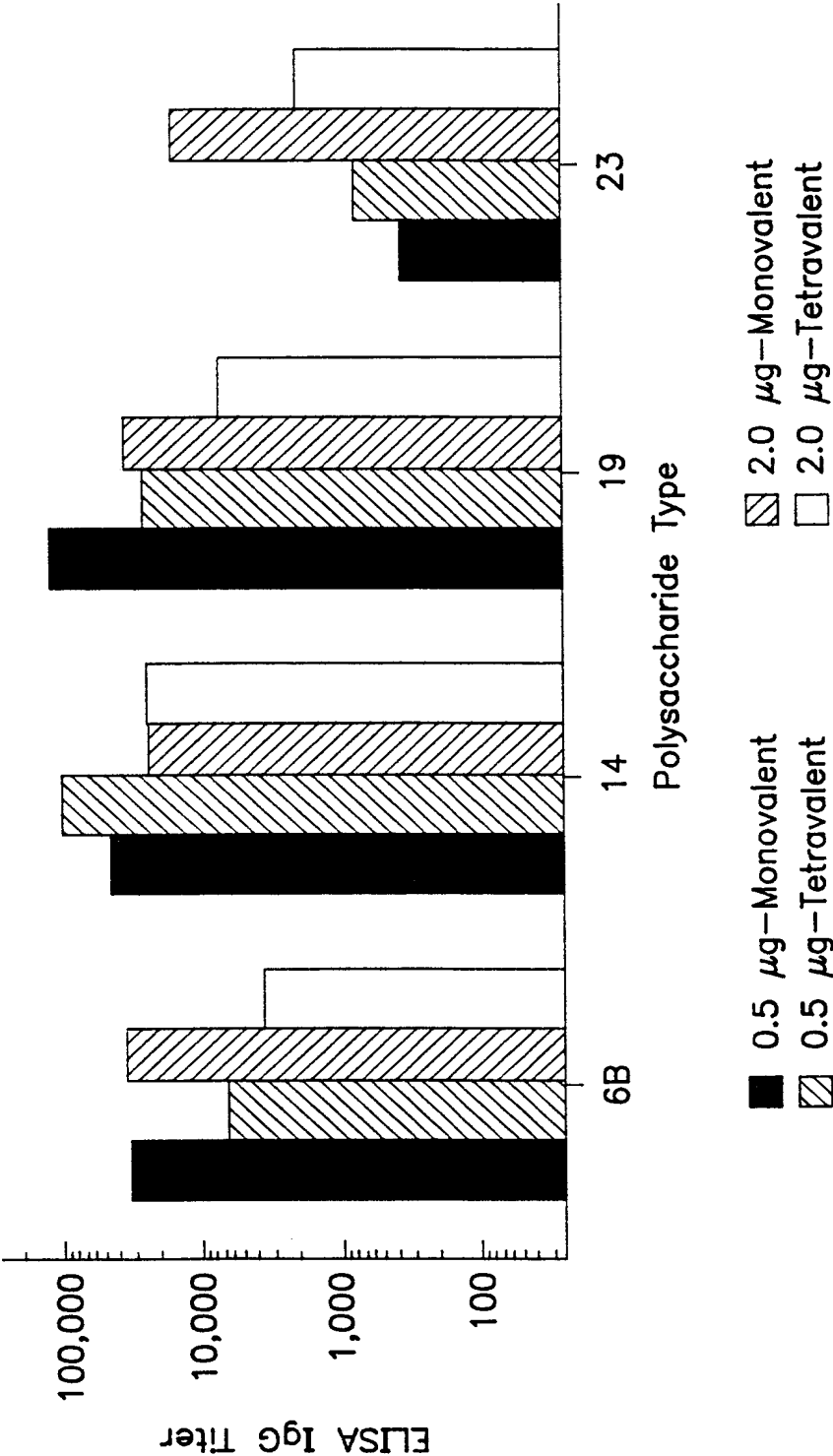


FIG. 8

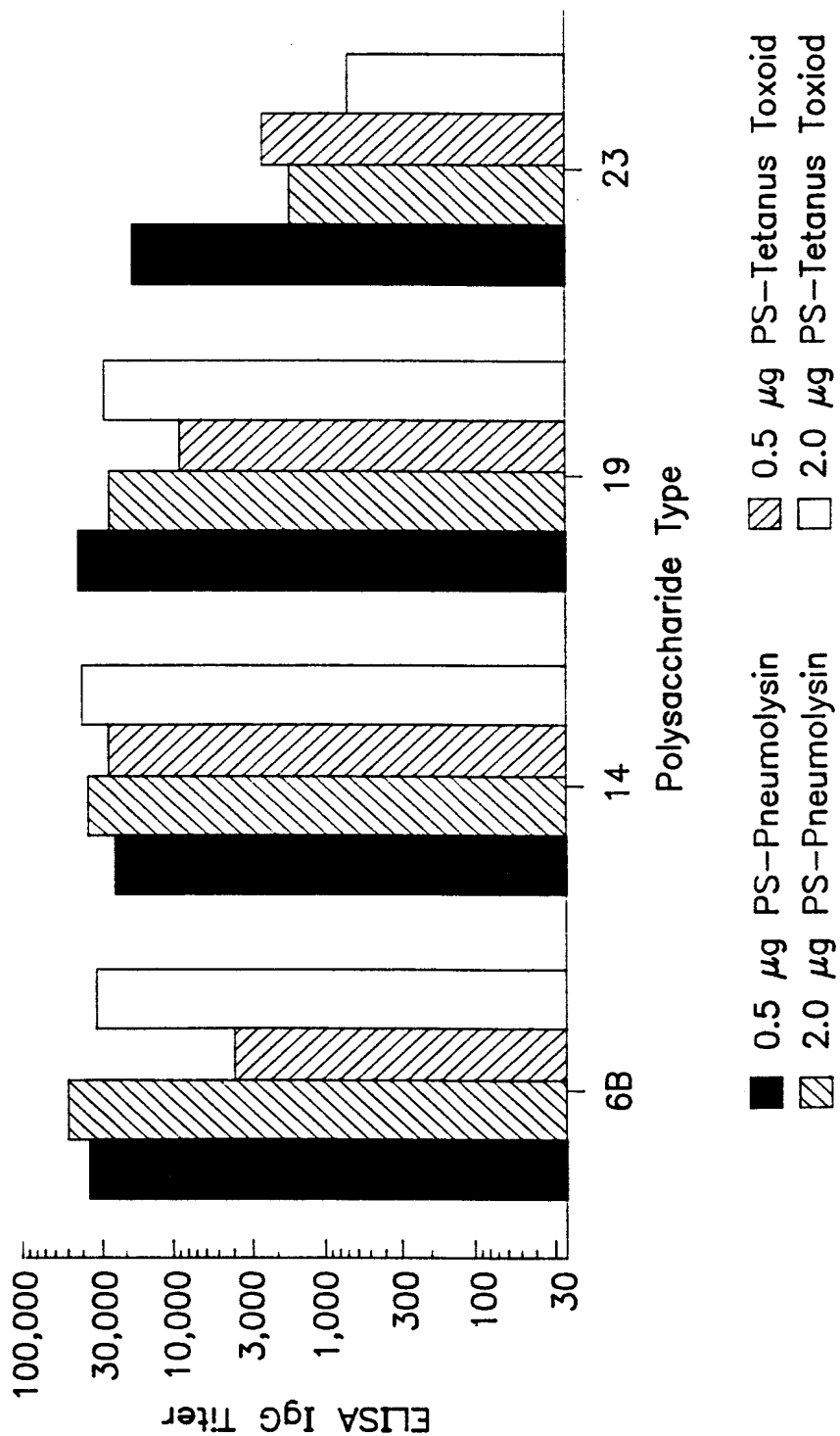


FIG. 9

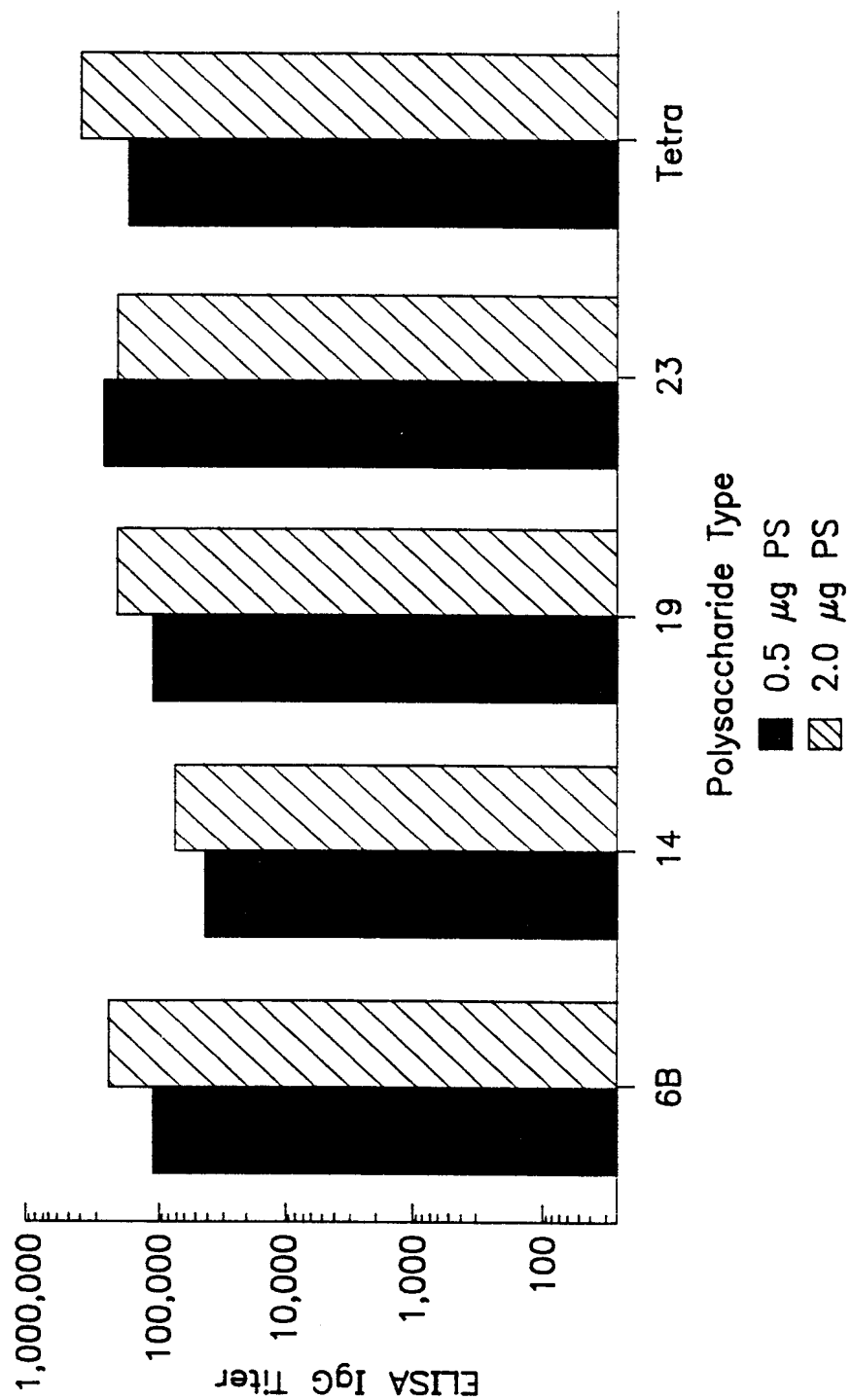


FIG. 10

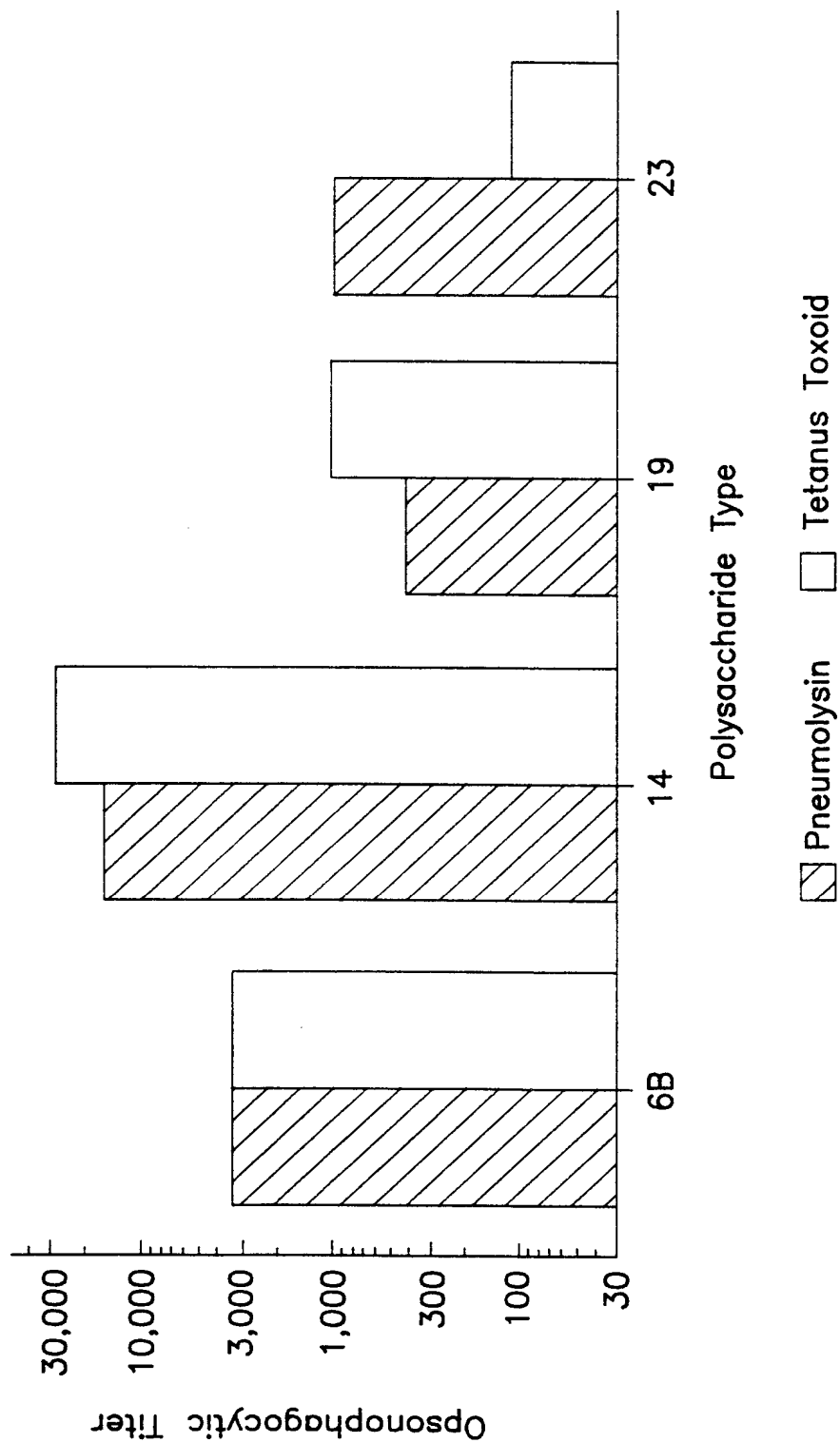


FIG. II

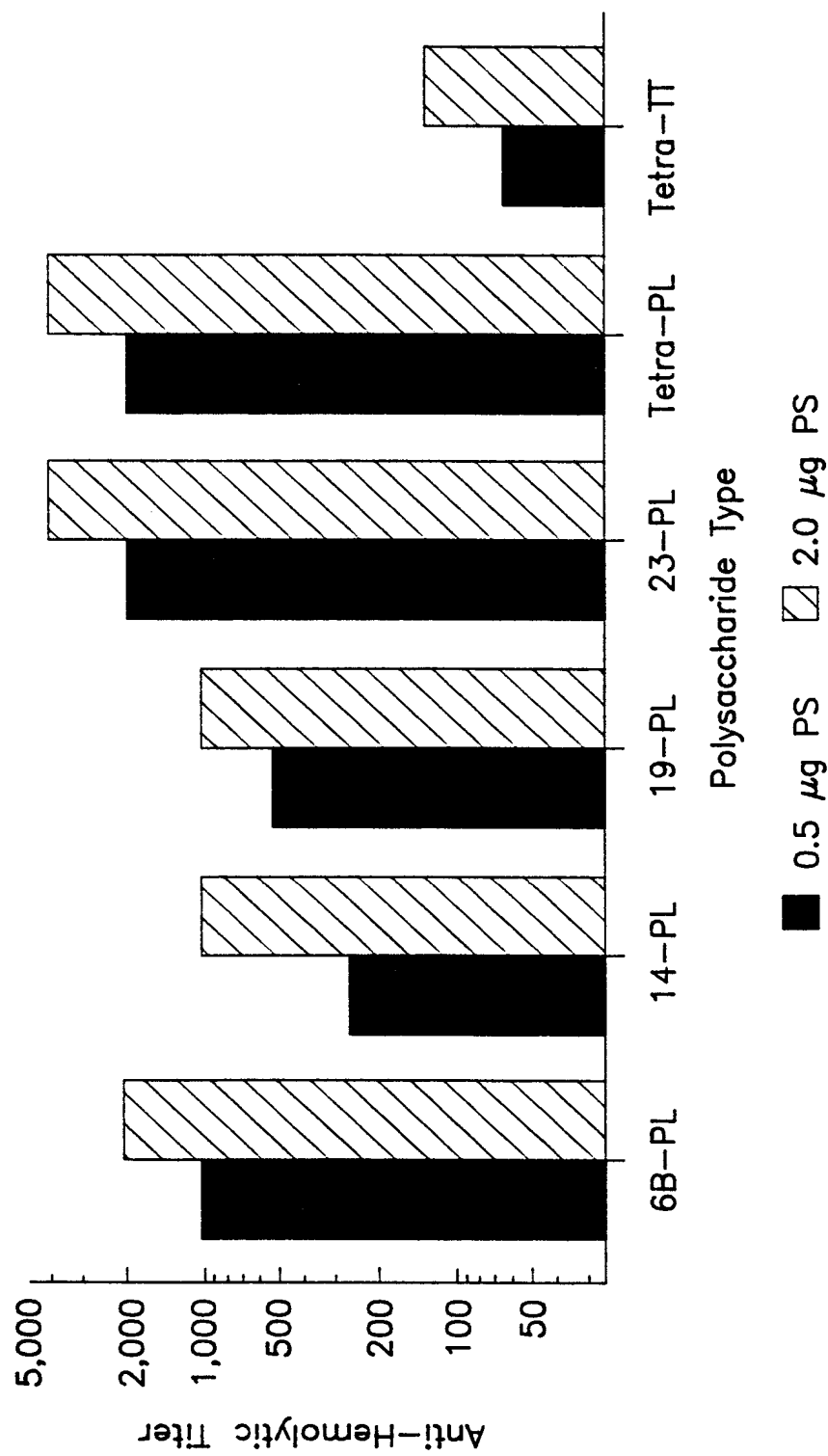


FIG. 12

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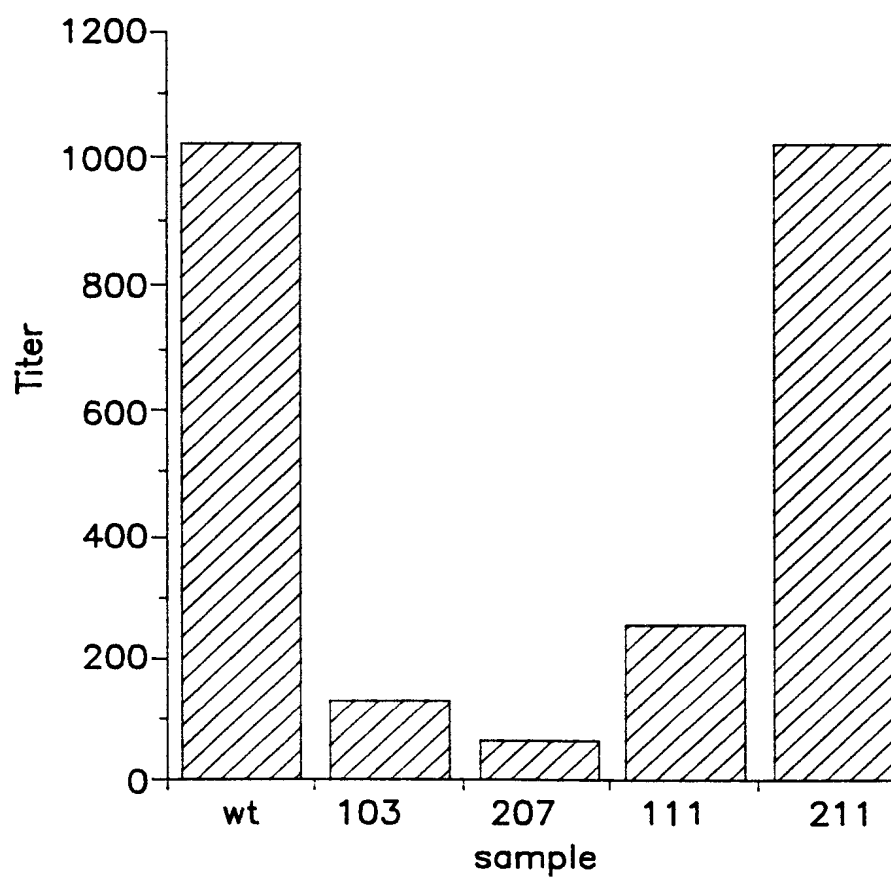


FIG. 13

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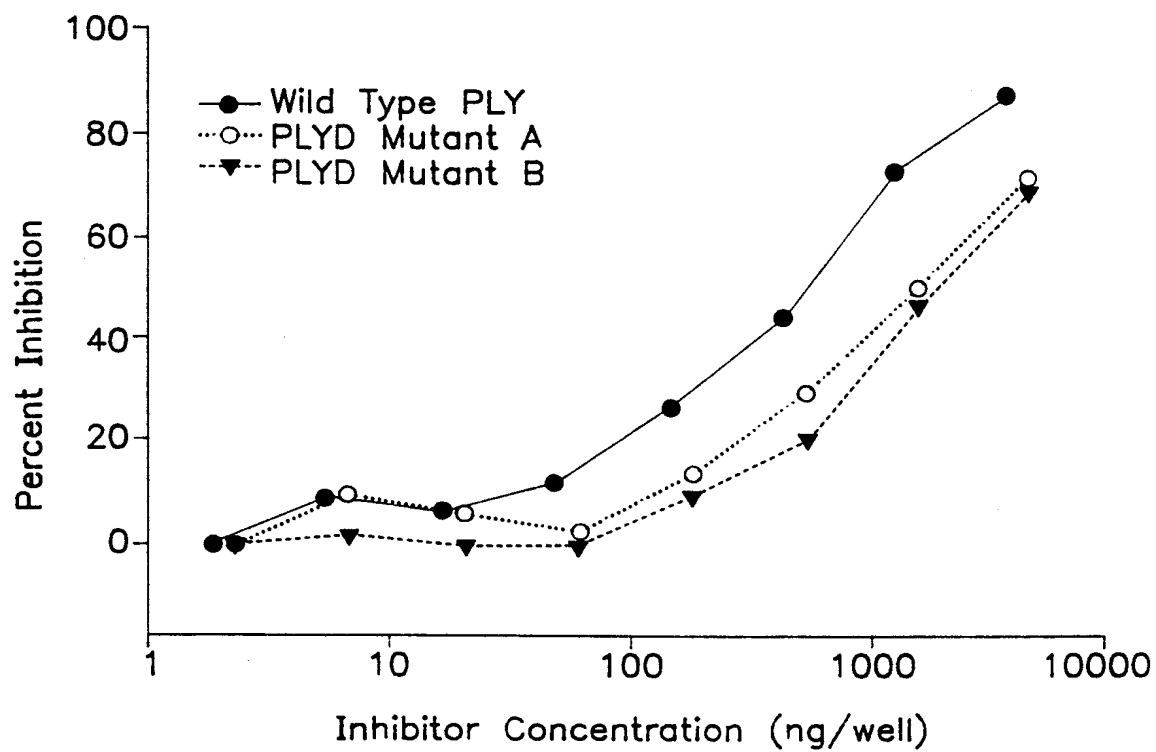
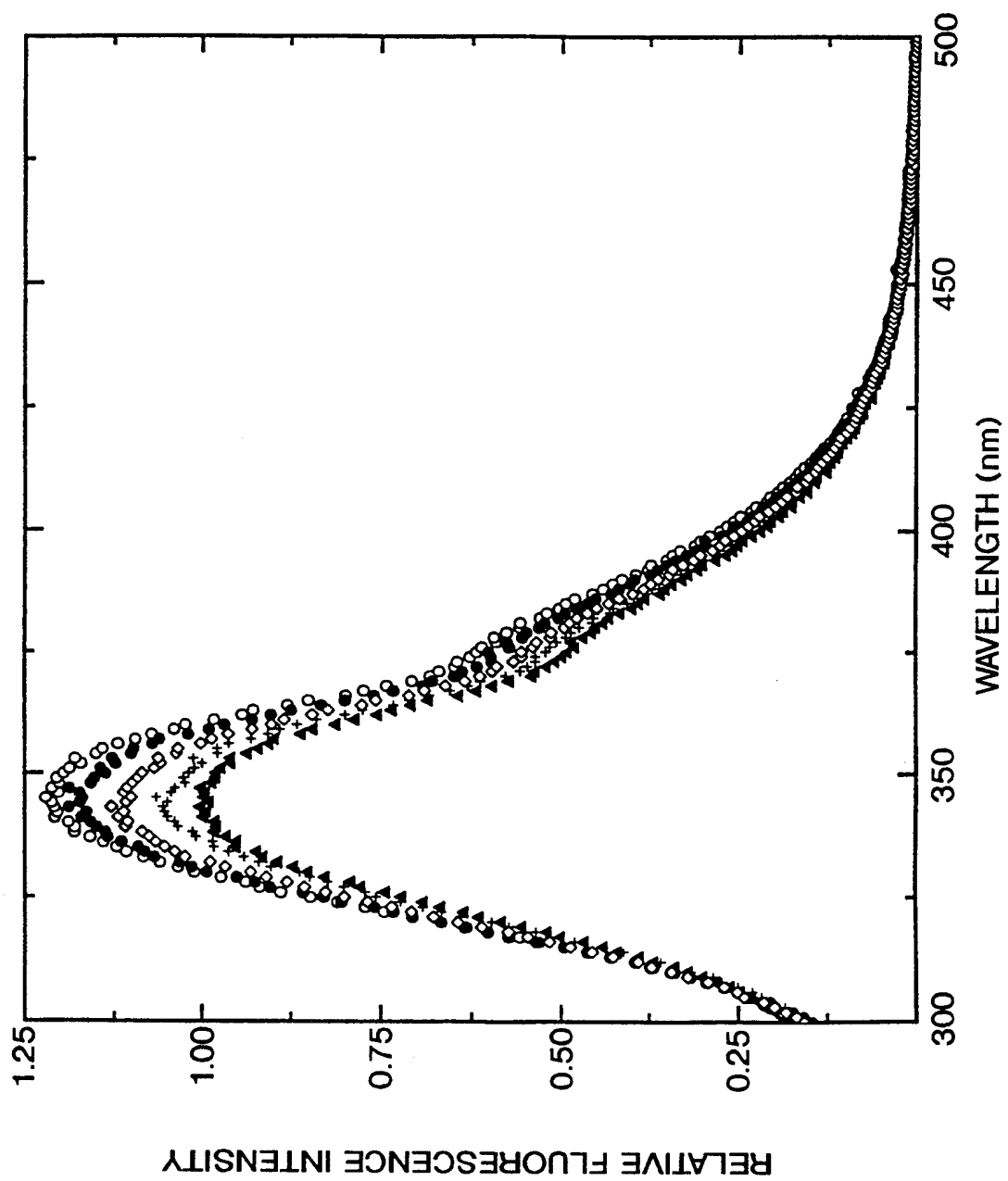


FIG. 14

**FIG. 15**



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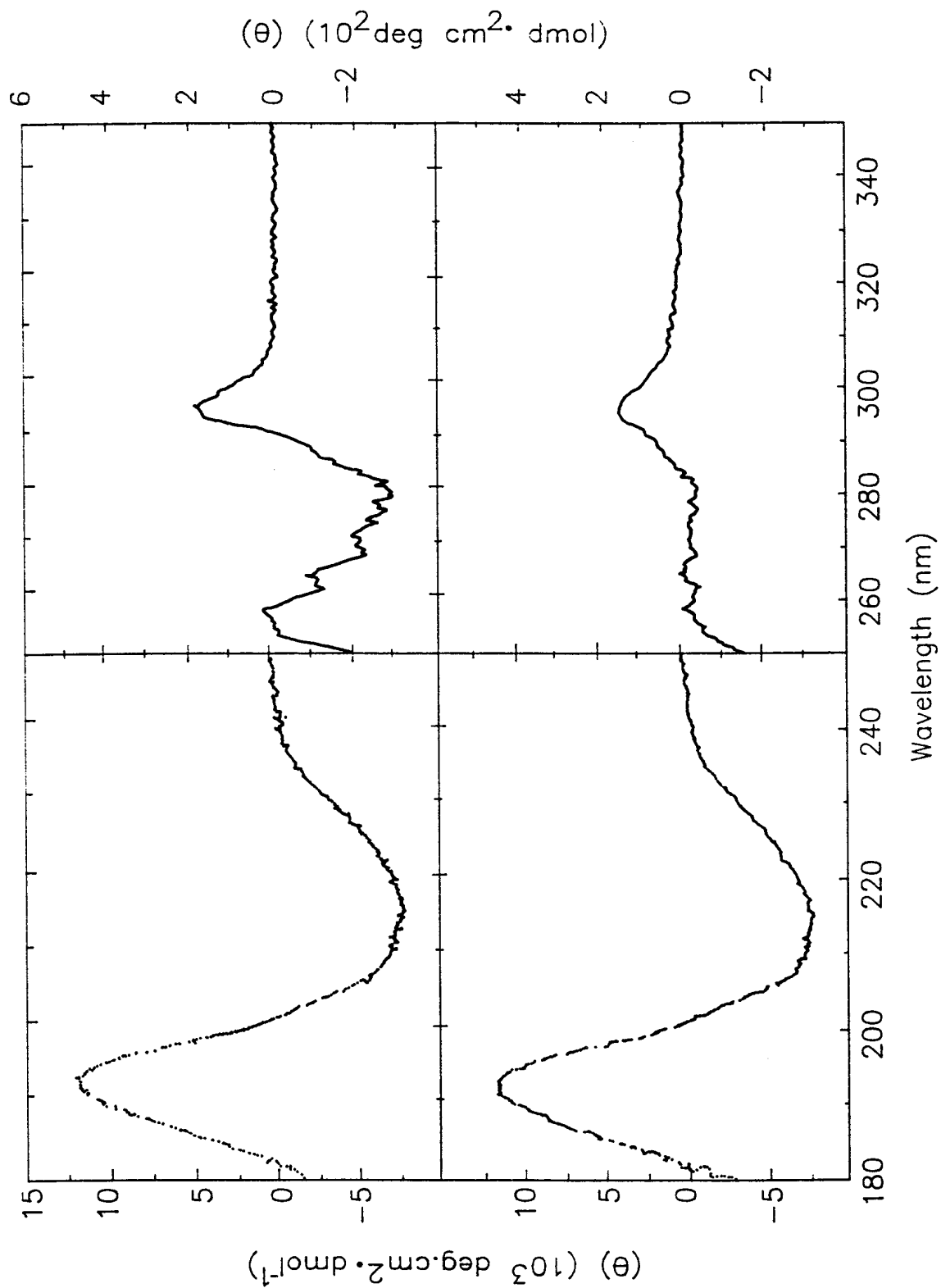


FIG. 16

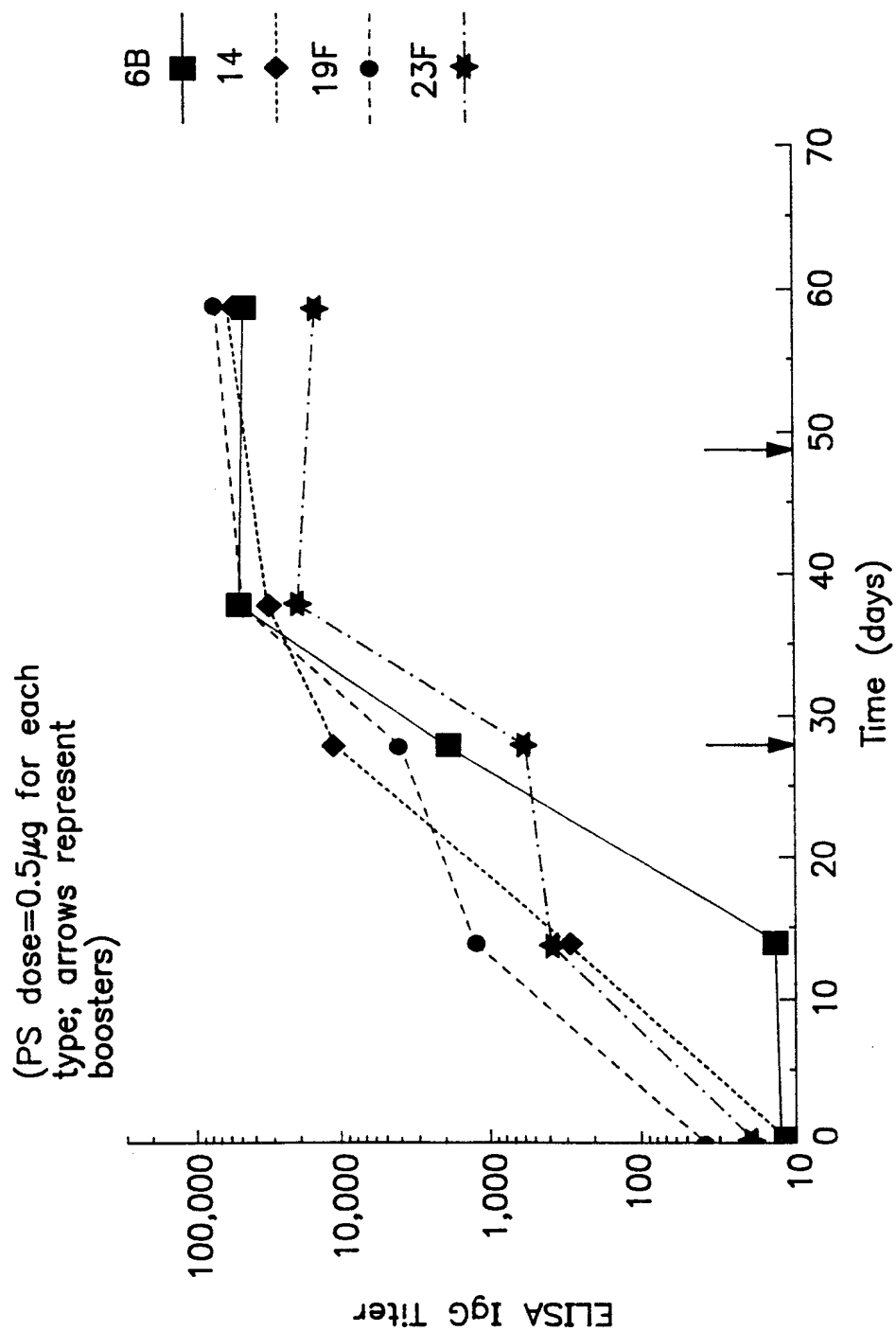


FIG. 17A

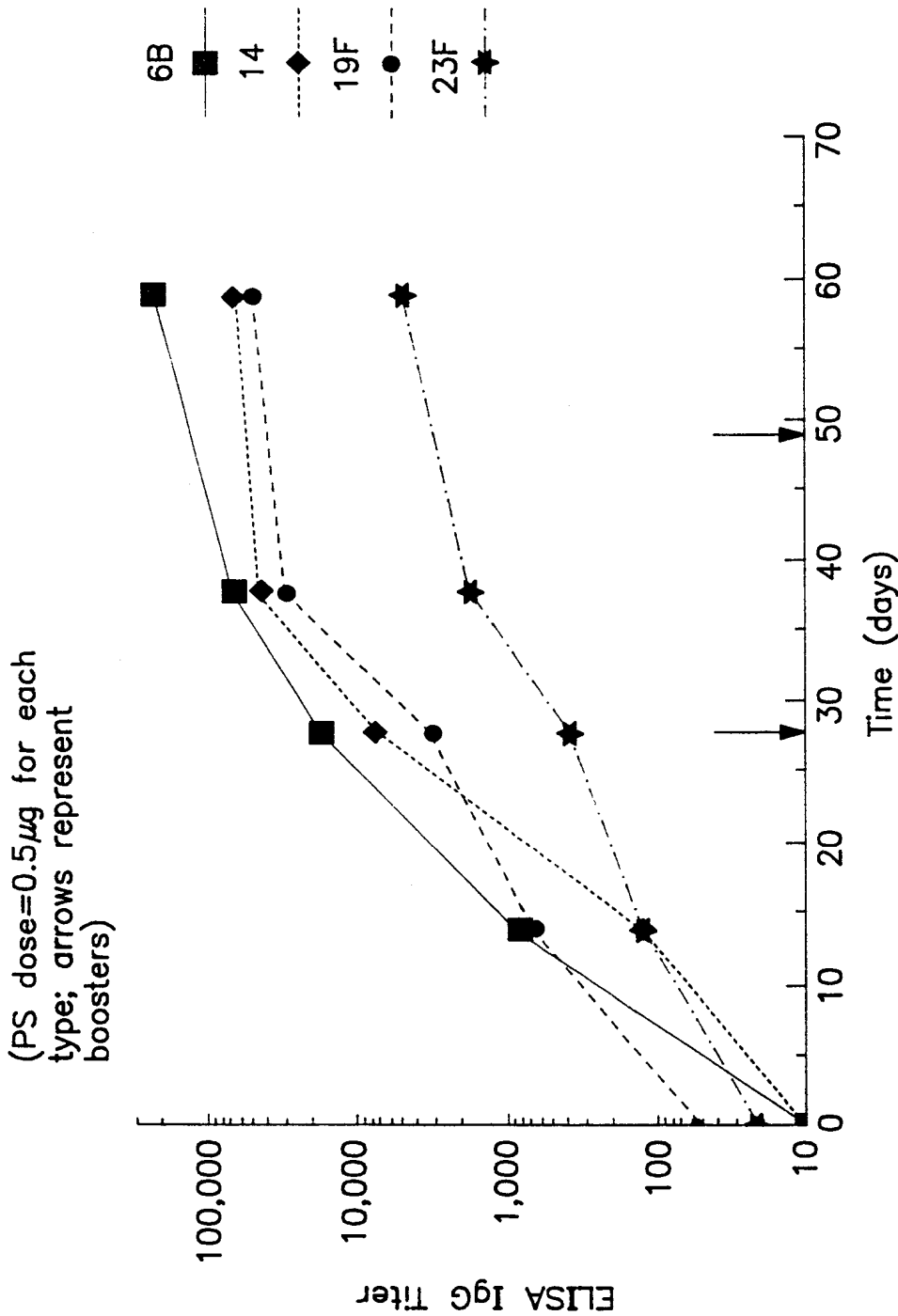


FIG. 17B

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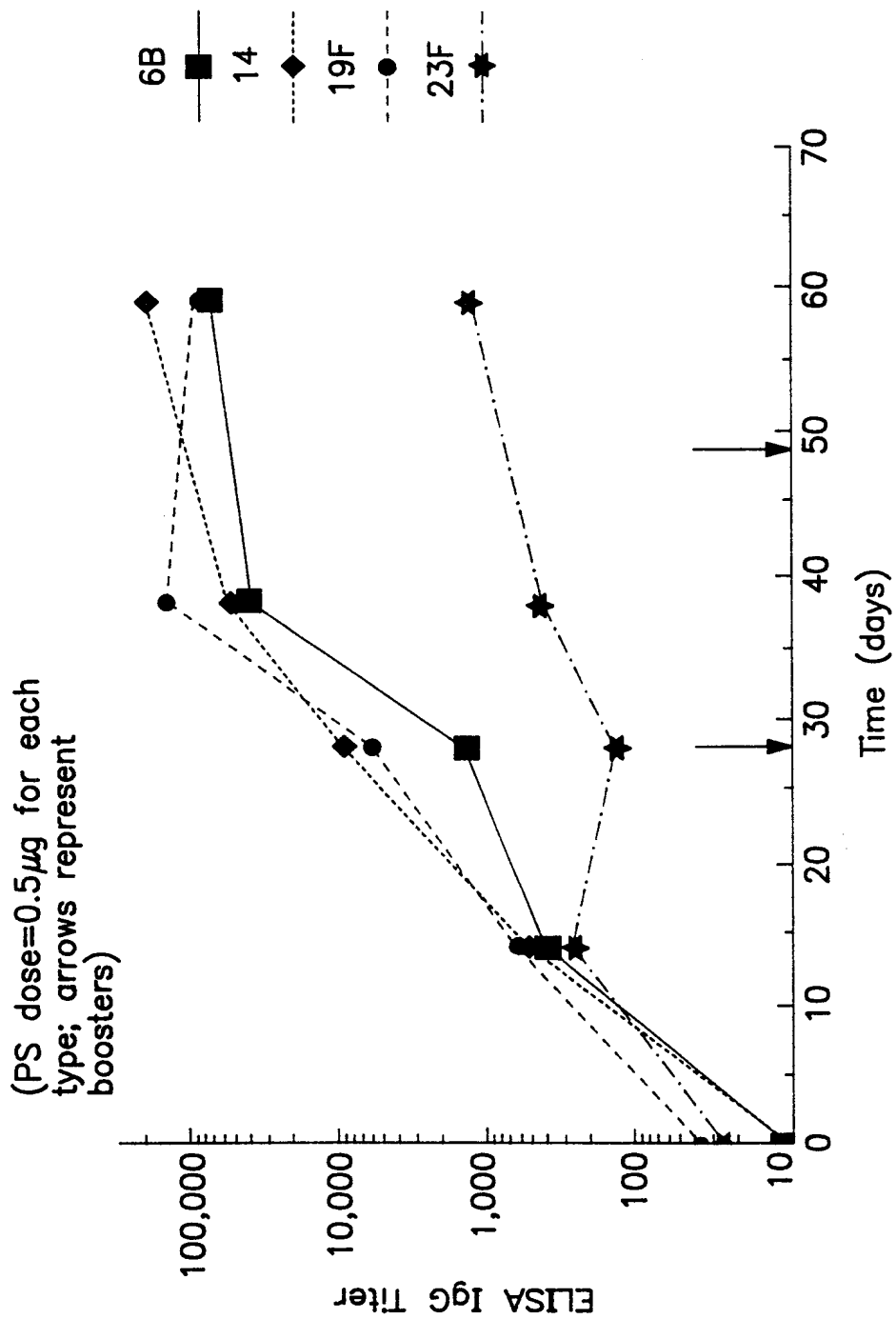


FIG. 17C



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 15/11, C07K 14/315, C07K 16/20, A61K 39/09		A3	(11) International Publication Number: <b>WO 99/03884</b>
			(43) International Publication Date: 28 January 1999 (28.01.99)
(21) International Application Number: PCT/US98/14716		POLVINO-BODNAR, Maryellen [US/US]; 621 Rolling Dale Road, Annapolis, MD 21401 (US). LIANG, Shu-Mei [US/US]; 6627 River Road, Bethesda, MD 20817 (US). TAI, Joseph, Y. [US/US]; 1370 Cinnamon Drive, Port Washington, PA 19034 (US).  (74) Agents: RADY, Arnold, I. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).  (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 21 July 1998 (21.07.98)			
(30) Priority Data:			
60/053,306	21 July 1997 (21.07.97) US		
60/073,456	2 February 1998 (02.02.98) US		
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US 60/053,306 (CIP) Filed on 21 July 1997 (21.07.97) US 60/073,456 (CIP) Filed on 2 February 1998 (02.02.98)			
(71) Applicant (for all designated States except US): NORTH AMERICAN VACCINE, INC. [CA/US]; 12103 Indian Creek Court, Beltsville, MD 20705 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): MINETTI, Conceicao [BR/US]; 3904 Isbell Street, Silver Spring, MD 20306 (US). MICHON, Francis [CA/US]; 4401 Rosedale Avenue, Bethesda, MD 20814 (US). PULLEN, Jeffrey, K. [US/US]; 6928 Garland Lane, Columbia, MD 21045 (US).		Published With international search report.	
		(88) Date of publication of the international search report: 8 April 1999 (08.04.99)	
(54) Title: MODIFIED IMMUNOGENIC PNEUMOLYSIN, COMPOSITIONS AND THEIR USE AS VACCINES			
(57) Abstract			
<p>This invention relates to modified pneumolysin polypeptides that retain the immunogenic nature of pneumolysin but have reduced or undetectable hemolytic activity compared to native pneumolysin. The invention also provides a method for generating novel pneumolysin variants with these desired characteristic properties. The invention also provides immunogenic compositions useful as pharmaceutical compositions including vaccines in which non-toxic, modified pneumolysin is used to stimulate protective immunity against <i>Streptococcus pneumoniae</i>. The vaccines may be compositions in which the modified pneumolysin is conjugated to bacterial polysaccharides or may be carried on an attenuated viral vector. In addition, the invention also provides a method of using the non-toxic, modified pneumolysin toxoid in order to stimulate antibodies against <i>Streptococcus pneumoniae</i> in a treated individual which are then isolated and transferred to a second individual, thereby conferring protection against <i>Streptococcus pneumoniae</i> in thesecond individual.</p>			

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/14716

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C07K14/315 C07K16/20 A61K39/09

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 90 06951 A (PATON JAMES CLELAND ET AL.) 28 June 1990 cited in the application see the whole document ---	1-3, 17, 22-34
A	WO 96 05859 A (AMERICAN CYANAMID CO) 29 February 1996 see abstract ---	26-34
A	WO 95 16711 A (FERNANDEZ JESUS M ;LOS TOYOS JUAN R DE (ES); FLEITES ANA (ES); UNI) 22 June 1995 see abstract see claims --- -/-	31-34



Further documents are listed in the continuation of box C.



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Date of the actual completion of the international search

7 January 1999

Date of mailing of the international search report

25/01/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
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Authorized officer

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# INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>MICHON F. ET AL.: "Multivalent pneumococal capsular polysaccharide conjugate vaccines employing genetically detoxified pneumolysin as a carrier protein" VACCINE, vol. 16, no. 18, November 1998, pages 1732-1741, XP002089380 UK see the whole document -----</p>	1-3



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/14716

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9006951 A	28-06-1990	AU 626961 B AU 4756490 A BG 51358 A CA 2005704 A DK 115591 A EP 0449856 A JP 4503948 T	13-08-1992 10-07-1990 15-04-1993 16-06-1990 14-08-1991 09-10-1991 16-07-1992
WO 9605859 A	29-02-1996	US 5565204 A AU 3363695 A CA 2198251 A EP 0778781 A JP 10504717 T	15-10-1996 14-03-1996 29-02-1996 18-06-1997 12-05-1998
WO 9516711 A	22-06-1995	ES 2075803 A ES 2088820 A EP 0687688 A	01-10-1995 16-09-1996 20-12-1995